

# IOWA STATE UNIVERSITY

## Digital Repository

---

Graduate Theses and Dissertations

Iowa State University Capstones, Theses and  
Dissertations

---

2011

## Pathogenesis and amelioration of nontyphoidal Salmonella encephalopathy in cattle infected with Salmonella enterica serovar Saintpaul

Nalee Xiong

*Iowa State University*

Follow this and additional works at: <https://lib.dr.iastate.edu/etd>

 Part of the [Medical Sciences Commons](#)

---

### Recommended Citation

Xiong, Nalee, "Pathogenesis and amelioration of nontyphoidal Salmonella encephalopathy in cattle infected with Salmonella enterica serovar Saintpaul" (2011). *Graduate Theses and Dissertations*. 11987.  
<https://lib.dr.iastate.edu/etd/11987>

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact [digirep@iastate.edu](mailto:digirep@iastate.edu).

**Pathogenesis and amelioration of nontyphoidal *Salmonella*  
encephalopathy in cattle infected with *Salmonella enterica* serovar  
Saintpaul**

by

**Nalee Xiong**

A dissertation submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Neuroscience  
Program of Study Committee:  
Steve A. Carlson, Major Professor  
Timothy A Day  
Michael Kimber  
Heather Greenlee  
Ronald Griffith

Iowa State University

Ames, Iowa

2011

Copyright © Nalee Xiong, 2011. All rights reserved.

## **DEDICATION**

I would like to dedicate this dissertation that is the graduate school culmination of my intellectual growth and unending curiosity toward life to my family. To my parents, who pushed me to be better than my best and who continuously provided the strength to believe in myself. And to my sister, Amy, who is the pinnacle of soundness and unfailingly offered me a reason to keep going.

## TABLE OF CONTENTS

<b>LIST OF TABLES</b>	vi
<b>LIST OF FIGURES</b>	vii
<b>ACKNOWLEDGEMENTS</b>	viii
<b>ABSTRACT</b>	ix
<b>CHAPTER 1. OVERVIEW</b>	1
Introduction	1
Pathogenicity of <i>Salmonella</i>	2
Salmonellosis	4
Typhoidal (enteric) bacterial infection	5
Nontyphoidal (gastroenteritis) bacterial infection	6
Virulence Factors	7
Virulence plasmids	7
Toxins	8
Protein secretion systems and SPIs	9
Two-component regulatory systems	10
Fimbriae	11
Flagella	11
Host Specificity	12
Host response	13
Macrophage survival	14
Neuropathogenic Strain of <i>Sstp</i>	15
Purpose	16
References	17
<b>CHAPTER 2. EVALUATION OF THE PATHOGENICITY AND VIRULENCE OF THREE STRAINS OF SALMONELLA ORGANISMS IN CALVES AND PIGS</b>	26
Abstract	26
Introduction	26
Materials and Methods	27
Animals, bacterial strain and preparation	27
<i>In vivo</i> infection-stress experiments involving neuropathogenic strains	28
Bacteriologic-based detection of <i>Salmonella</i> spp	29
Results	30
Discussion	30

References	31
<b>CHAPTER 3. EXPRESSION OF A COLLAGENASE THAT ENABLES BLOOD-BRAIN BARRIER PENETRATION FOR SALMONELLA IMPLICATED IN BOVINE ENCEPHALOPATHIES</b>	33
Abstract	33
Introduction	34
Materials and Methods	35
Bacterial strains	35
<i>In vitro</i> experiments assessing <i>clg</i> derepression	35
<i>In vivo</i> experiments	36
Qualitative <i>Salmonella</i> assessment	37
Results	37
Assessment of host neuroendocrine hormones on <i>clg</i> expression	37
Repression of <i>clg</i> prevented <i>Sstp</i> NPG from traversing the BBB	38
Cilostazol prevents <i>Sstp</i> NPG from traversing the BBB	38
Discussion	39
References	40
<b>CHAPTER 4. BETA-LACTAM ANTIBIOTICS PREVENT SALMONELLA- MEDIATED ENCEPHALOPATHY REGARDLESS OF BETA-LACTAM RESISTANCE</b>	44
Abstract	44
Introduction	45
Materials and methods	47
Bacterial strains used and preparation	47
<i>In vivo</i> experiments	48
Qualitative isolation of <i>Salmonella</i>	49
Semi-quantification of GLT-1	49
Results	50
Assessment of neurologic disease in <i>Sstp</i> NPG-infected calves treated with beta-lactam antibiotics	50
Assessment of Glut-1 expression in <i>Sstp</i> NPG-infected calves treated with beta-lactam antibiotics	51
Assessment of neurologic disease in <i>Sstp</i> NPG-infected calves treated with glutamate antagonist	51
Assessment of neurologic disease in <i>Sstp</i> NPG-infected calves treated with non-beta-lactam antibiotics	51
Discussion	52
References	53

<b>CHAPTER 5. THE LIPOPOLYSACCHARIDE OF <i>SALMONELLA ENTERICA</i> SEROVAR SAINTPAUL PLAYS A ROLE IN THE INDUCTION OF NONTYPHOIDAL <i>SALMONELLA</i> ENCEPHALOPATHY IN THE BOVINE</b>	58
Abstract	58
Introduction	58
Materials and methods	61
Bacterial strains	61
<i>In vivo</i> experiment	61
Bacteriologic-based detection of <i>Sstp</i> NPG	62
Results	63
Discussion	64
References	65
<b>CHAPTER 6. GENERAL CONCLUSIONS</b>	70
Overview	70
Host-condition specific phenomenon	70
Collagenase allows <i>Sstp</i> NPG to penetrate blood-brain barrier	72
<i>clg</i> is depressed by bovine stress factors	72
NTSE requires production of <i>clg</i> to traverse BBB	73
<i>clg</i> allows for a paracellular route into the brain	74
Beta-lactam antibiotics ameliorates neurologic disease	74
Beta-lactam antibiotics serve as a viable neuroprotective agent	75
Glutamate plays a role in neurologic disease	76
Beta-lactam antibiotics are neuroprotective through increased GLT-1 expression	77
Lipopolysaccharides provoke neurologic disease	77
LPS plays a role in the induction of neurologic disease	78
Potential role of LPS in producing neurologic signs	79
Future directions	80
Investigating the effects of traversing the BBB	80
Determining the role of LPS	80
References	81
<b>APPENDIX</b>	85

## LIST OF TABLES

Table 1.1	Comparison between bacterial endotoxins and exotoxins	22
Table 1.2	Basic chemistry of a two-component phosphoryl transfer signal transduction pathway	23
Table 2.1	Qualitative assessment of <i>Salmonella</i> organisms	32
Table 3.1	Summary of results for calves infected with <i>Salmonella</i>	42
Table 4.1	Summary of <i>in vivo</i> experimental group treatment set up	56
Table 5.1	Summary of results for calves treated for LPS blocking	69

## LIST OF FIGURES

Figure 1.1	Entrance into Hep-2 cell via modification of host actin cytoskeleton	24
Figure 1.2	Possible trafficking of lysosomes to SCV	25
Figure 3.1	Expression of <i>clg</i> in <i>Sstp</i> NPG exposed to bovine serum	43
Figure 4.1	Assessment of <i>Salmonella</i> blood and brain culture, GLT-1 hyperexpression, and neurologic signs in calves	57



## ACKNOWLEDGEMENTS

I would like to extend my warmest gratitude to my advisor, Dr. Steve Carlson, for sharing his extensive knowledge and providing kind guidance throughout my doctoral career. He has served as my touchstone for what it truly means to be a researcher. I want to offer a special thanks to Dr. Michael Kimber for his patience and support as a professor and scientist who welcomed all queries and technical assistance. Drs. Tim Day, Heather Greenlee and Ronald Griffith have distinctly impacted the development of my academic career through their insight and input for which I am most grateful.

Acknowledgement also needs to be given to the many colleagues that took part in my research and assisted in the obtainment of data for the progression of this work. I am grateful to Matthew Brewer, Katherine Weeks, Dr. Gwyneth Watrous, and Dr. Alison Barnhill for their veterinary expertise and animal handling during my *in vivo* studies. I am also thankful for the bioinformatic assistance I received from Mostafa Zamanian and the helpful ideas provided through discussions. And lastly, I express my sincere gratitude to Brock Young for his scientific wisdom, intuitive counsel, and unwavering solace that have been instrumental to the finalization of my research.

## ABSTRACT

*Salmonella enterica* serovar Saintpaul (*Sstp*NPG) is a unique isolate capable of producing bovine encephalopathy in the presence of host neuroendocrine hormones. *In vivo* and *in vitro* molecular techniques were used to determine how specific the conditions are for the perpetuation of neuropathogenicity, to understand how the strain traversed the blood-brain barrier (BBB), to find a potential pharmacological agent that abrogated the effects of neuropathogenicity, and to assess the bacterial factors that provoke neurologic disease. In order to determine if collagenase (*clg*) is responsible for the traversal of the BBB, RT-PCRs were conducted to determine *clg* expression. Results elucidated a unique relationship between bovine stress factors and *clg* that could be abrogated by cilostazol, which is capable of tightening the BBB. Knock out of *clg* also abolished signs of neurologic disease in calves. It was also established that these neurologic deficits seen in cattle could not be reproduced in swine. In order to determine if these signs of neurologic disease could be abolished, beta-lactam antibiotics, which has been recently determined as a novel neuroprotective agent through the upregulation of glutamate transporter expression, were given as a treatment and successfully ameliorated bovine encephalopathy indicating excess extracellular glutamate as a key component. Since little CNS inflammation occurred, neurologic disease most likely resulted from signal transduction aberrations. Lipopolysaccharides (LPS) could be possible elements that provoke such effects. Considering this, LPS was blocked and/or debilitated pharmacologically, resulting in no

signs of neurologic disease in calves and alluding to a potential provoker of bovine encephalopathy.

## CHAPTER 1. OVERVIEW

### INTRODUCTION

*Salmonella* are Gram-negative, rod-shaped bacteria possessing peritrichous flagella for motility. These facultative anaerobes are capable of producing ATP through aerobic respiration if oxygen is present but are also able to switch to fermentation in its absence. They also obtain energy from organic compound sources such as glucose, categorizing them as chemoorganotrophs. The genus *Salmonella* is comprised of two species that are 95-99% homologous on a DNA level, *S. bongori* and *S. enterica* [18]. *S. enterica* can be further delineated into six subspecies [9]. Subspecies *enterica* (I) is mostly isolated from avian species and mammals, while *S. bongori* and *S. enterica* subspecies *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) are typically found in cold-blooded vertebrates [7, 14].

Demarcation into serotypes derive from variance found within the O antigen of the lipopolysaccharide (LPS), the H antigen of the flagella, and oftentimes the capsular polysaccharides (Vi) antigens using the Kauffman-White scheme [9, 14, 34]. Subtyping of *Salmonella* strains are conducted through the following molecular techniques: pulsed-field gel electrophoresis, amplified fragment length polymorphism, ribotyping and multi-locus sequence typing [1, 39]. Plasmid profiling, arbitrarily primed polymerase chain reaction (PCR) fingerprinting, and repetitive-sequence-based PCR fingerprinting techniques are also used to identify strains into serotypes [2]. Specifically, serotypes phenotypically characterize strains based on the immunologic

reactivity of O, H, and Vi antigens. Determining the species and subspecies into which a strain falls through serotyping is important for better investigation of outbreaks because certain diseases or epidemiology is often in association with certain serovars.

*S. enterica* serovar Saintpaul (*Sstp*) is an uncommon serotype and has only been reported as a bacterial strain of infection in 1.6% of cases annually [19]. This strain is typically associated with foodborne illnesses resulting from meat consumption. Recently, *Sstp* has been implicated in *Salmonella* outbreaks from consumption of multiple fresh produce such as bean sprouts, lettuce, tomatoes, and melons [19]. During the summer of 2008, one of the largest *Salmonella* outbreaks resulted from *Sstp*-infected jalapeño peppers [19].

#### PATHOGENECITY OF *SALMONELLA*

*Salmonella* infection is a result of ingesting food or water that has been contaminated. The first barrier pathogens have to overcome is the intestinal lining of the host, more specifically M (microfold) cells. Enterocytes are also infected by *Salmonella* in bovines [27]. These interactions occur fairly quickly. Frost and colleagues (1997) conducted experiments implicating that 5 minutes post-inoculation resulted in bacterial migration to M-cells and host response of M-cell engulfing bacteria. M-cells are specialized antigen epithelial cells of the follicle-associated epithelium covering the domed villi of the Peyer's patch in the small intestine and have an immunological role through the transportation of foreign material from the lumen to lymphoid tissues for the purpose of immunological sampling [11]. Their role is

comparable to that of immunological surveillance -- a feature often exploited by pathogens in order to invoke systemic effects [17, 18]. M-cells are polarized and form tight junctions, delineating the apical and basolateral membrane domains [17]. The apical membrane facilitates the adherence and uptake of antigens and microorganisms. Instead of possessing a brush border, it has varying microvilli integrated with large plasma membrane subdomains exposed to the lumen, which leads to a desirable pathway through the intestinal layer for *Salmonella*. The physical structure of M-cells is maintained by a dense scaffold of intermediate filaments that form around the nucleus and intraepithelial pocket, a unique element to the cell.

Rearranging the cytoskeletal structure of M-cells induces the internalization of *Salmonella*. This modification results in cytotoxicity and augments the persistence of the infection via contiguous enterocytes. Through modification of host actin cytoskeleton and macropinocytosis, *Salmonella* invade cells and enter what is referred to as a *Salmonella*-containing vacuole (SCV) where they can proliferate (see Figure 1). The generation of SCV, also known as a phagosome, is a result of bacteria coming into contact with the surface of the target host cell and thereby engulfed to form the vacuole [5, 18]. Specifically, bacteria attach to the apical epithelial surface which perpetuates the release of effector proteins that go onto rearrange the cytoskeleton to form membrane ruffles that then reach out and encapsulate the bacteria attached to the surface into a large vesicle, the SCV [25]. Bacteria are confronted with nutrient-limiting conditions, low pH, cationic peptides and reactive oxygen species (ROS) inside the SCV

[27]. The SCV then migrates to the basolateral membrane and the apical epithelial surface reforms.

After traversing the intestinal cell, *Salmonella* enter macrophages, which occur either through phagocytosis or through the release of effector proteins that will then mediate bacterial engulfment [18, 25]. Bacteria are capable of being engulfed by macrophages in the lamina propria within 60 minutes [11]. Survival within macrophages is essential for establishing systemic infection and for host adaptation. From this advantage, *Salmonella* can proliferate throughout the host with the migration of infected macrophages into other organs (*e.g.*, liver, spleen, and very rarely the brain) within the reticulo-endothelial system. The ability to withstand the harsh environment of macrophages will be further discussed.

## SALMONELLOSIS

Salmonellosis can manifest as gastroenteritis or systemic disease characterized by septicemia (*i.e.*, typhoid or paratyphoid fever) [9]. On occasion, salmonellosis can also produce bacteremia, whereby bacteria can be isolated from the blood, often resulting in high fevers (distinguishable from typhoid fevers, which is continual throughout the infection), and is quickly cleared from the host [32]. Over 16 million cases of typhoid fever have been reported worldwide each year, with approximately 600,000 fatal outcomes, and there have also been 1.3 billion incidences of gastroenteritis caused by non-typhoidal salmonellosis resulting in 3 million deaths annually [13]. In regard to human infections, *Salmonella enterica* can be categorized as

either typhoidal (*S. Typhi*, *S. Paratyphi A*, *S. Paratyphi B*, *S. Paratyphi C*, *S. Choleraesuis*, and *S. Sendai*) or nontyphoidal *Salmonella* (NTS)[14]. In cases where host immune responses are attenuated, NTS are capable of systemic infections, though this is typically uncommon. Even more rare is NTS that produces encephalopathy in hosts like that of a neuropathogenic strain of *Sstp*.

#### *Typhoidal (enteric) bacterial infection*

Typhoid fever is a systemic disease that results in the colonization of the lymph nodes, liver, kidney and blood of hosts. This infection is caused by strains of *Salmonella* that are specific to humans or higher primates, such as *Salmonella Typhi* and *Paratyphi*. Infection with *S. Typhi* is significantly more common than with *S. Paratyphi* but the clinical signs for each are indistinguishable [28]. In chronic cases, *Salmonella* can also be isolated from the gall bladder. Signs of this infection include fever, abdominal pain, transient diarrhea or constipation, and maculopapular rash [25]. These strains have an incubation period lasting between 5 to 9 days and symptoms can subsist for three weeks [28].

Meningitis and other forms of encephalopathy can also result from typhoidal bacterial infection, especially in young children, indicating *Salmonella's* ability to traverse the blood-brain barrier (BBB). When left untreated, 10 to 15% cases of typhoidal bacterial infection end in death [25]. As mentioned previously, this disease is typically specific to humans infected by *S. Typhi* and *S. Paratyphi* but *S. Typhimurium* has been demonstrated to produce typhoid-like fever in mice.



### *Nontyphoidal (gastroenteritis) bacterial infection*

Unlike typhoidal bacterial infections, nontyphoidal bacterial infections produce gastroenteritis in multiple hosts – such as avian, bovine and porcine hosts.

Gastroenteritis can result from any of the more than 2,500 *Salmonella* serovars, though it is most commonly associated with *Salmonella* Typhimurium and *Salmonella* Enteritidis [32]. Inflammatory responses that lead to intestinal pathology and diarrhea signify gastroenteritis [18]. NTS strains have a brief incubation period lasting between 12 to 72 hours before the onset of symptoms occur, which typically lasts no longer than 10 days [28]. The limited dissemination of infection is indicative of an inability to overcome host defenses outside the intestinal mucosa.

Infection of cattle with *S. Typhimurium* typically results in gastroenteritis that is often signified by diarrhea. Signs of infection, including anorexia, fever, dehydration and prostration, typically occur within 12 to 48 hours [32]. Significant pathological changes occur in the calf intestine where the lymph nodes are enlarged and the small intestine (specifically, caudal jejunum and ileum) is congested and distended [32]. Increased neutrophil migration through the epithelium on top of *Salmonella* infiltration of neutrophils is considered the pathogenesis of diarrhea due to the event occurring prior to the intestinal lumen accumulating fluid [31]. Morphologic changes result in increased expression of CXC chemokines (GRO $\alpha$ , GRO $\gamma$ , and GCP2) and pro-inflammatory cytokines (IL-1 $\beta$  and IL-8) [31]. The enteric effect of *S. Typhimurium* in calves is similar to that of gastroenteritis in humans making it an ideal model to study.

## VIRULENCE FACTORS

The type of salmonellosis is determined by the relationship between the host and bacterium. This relationship rests on the ability to colonize and survive within the host. The pathogenesis of *Salmonella* is contingent on the ability to invade and evade host cells in order to survive and replicate within the environment. Virulence factors, such as virulence plasmids, toxins, fimbriae, and flagella allow the bacteria to colonize and survive the antimicrobial environment. Other virulence factors such as *Salmonella* Pathogenicity Islands (SPI), which contain secretion systems that allow the bacterium to penetrate phagocytic and non-phagocytic cells, also contribute to pathogenesis and bacterial persistence.

### *Virulence plasmids*

Only a few serovars within subspecies I, particularly those showing host adaptation, have virulence plasmids that are essential to bacterial multiplication within the reticulo-endothelial system of hosts [29]. Virulence plasmids are identified by a 7.8 kb region referred to as *spv* (*Salmonella* plasmid virulence), which houses five genes designated as *spv RABCD* [38]. Little is known of their function with the exception of *spvR*. The regulatory protein produced by *spvR* is responsible for the expression of other *spv* genes. Though the plasmid is putatively not involved in the enteric stages of disease, Libby and colleagues (1997) demonstrated that it does play a role in the severity of the enteric disease.

## *Toxins*

There are also two forms of toxins that aid in the success of bacterial invasion: endotoxins and exotoxins. Table 1 addresses additional differences between these two virulence factors. Endotoxins are found imbedded in the outer membrane of Gram-negative bacteria and most is often synonymous with LPS but any cell-associated bacterial toxin can be considered as such. Endotoxins can have an effect through release, which may occur during growth or bacterial lysis, or by remaining attached to the cell membrane and interacting with the host receptors. The toxicity of LPS typically results from the ability of lipid A to perpetuate potent and noxious immune responses, such as the release of cytokines [tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ )] and mammalian inducible nitric oxide synthase (iNOS). The release of these pro-inflammatory mediators is an indication of endotoxins causing harm to the host during salmonellosis while still attached to live bacteria [16].

Exotoxins are proteins (usually toxic) that modify the host environment and cell functions, such as cytoskeleton dynamics, vesicular trafficking, morphology of host plasma membrane, and immune responses in order to gain entry into targeted cells [33, 36]. Exotoxins are considered either cytotoxins or enterotoxins [38] and are distributed through a simple release into the cytoplasm, delivered to the target cell under the guidance of a chaperone protein or via protein secretion systems, such as type III secretion systems (T3SS) or type IV secretion systems (T4SS) that deliver the bacterial toxins in a spatial and temporal fashion [33].

### *Protein Secretion Systems and SPIs*

Seven different types of protein secretion systems (types I – VII) have been identified in *Salmonella enterica* [21]. T3SSs resemble that of a molecular syringe that delivers virulence proteins capable of manipulating host-cell functions (signal transduction, cytoskeletal rearrangement, membrane trafficking, and cytokine expression) for bacterial survival and proliferation. T3SS-1 mediates *Salmonella* invasion of epithelial cells and T3SS-2 is essential for macrophage survival [10, 28]. T6SS has also been indicated in bacterial infection, intracellular survival, biofilm formation, flagella regulation, quorum sensing, and stress response for *Salmonella enterica* [21]. *S. Typhimurium* and *S. Typhi* are capable of releasing proteins via T3SS that will provoke its own uptake into target host cells wherein it triggers a secondary T3SS that will release additional effectors in order to impair phagosome maturation, the generation of SCV, and prevent phagosome-lysosome fusion [3, 25]. This type of bacterial internalization occurs when membrane ruffles are formed as a result of cytoskeletal rearrangements caused by T3SS effectors. T3SS-2, located on SPI-2, is capable of preventing fusion of NADPH-dependent oxidase with SCV thereby inhibiting the production of reactive oxygen species (ROS) inside the vacuole. T3SSs are strongly conserved but the effector proteins released into host cytoplasm are highly variable, contributing to distinct virulence patterns [25].

T3SSs are regulated through *Salmonella* pathogenicity islands (SPIs), specifically SPI-1 and SPI-2 regulate T3SS-1 and T3SS-2 respectively, which are clusters of virulence genes located either on the bacterial chromosome or embedded in virulence

plasmids. To date, there have been 12 SPIs identified [18]. SPIs are horizontally-acquired genetic elements, whereby incorporation proceeds into non-progeny. Examples of horizontal gene transfer are transformations (uptake and expression of foreign genetic material), transductions (transfer of bacterial DNA from one bacterium to another via bacteriophages), and bacterial conjugation (transfer of genetic material through bacterial cell-to-cell contact). *Salmonella* are not naturally transformable and typically undergo horizontal gene transfer through transductions and conjugation. SPI-1 is essential to the initiation of bacterial infection and invasion of the intestinal tract, while SPI-2, which contains a second T3SS, is crucial for bacterial survival within host macrophages [13, 18, 36]. A 25 kb fragment containing four operons (regulatory, structural I, structural II, and effector/chaperone) responds to SPI-2 T3SS [10]. SPI-2 operons are transcriptionally regulated by a two-component regulatory system, SsrA/SsrB [10].

### *Two-Component Regulatory Systems*

Two-component regulatory systems allow bacteria to interact with their environment by sensing and responding to changes in external conditions [35]. Generally, two-component regulatory systems consist of a histidine protein kinase (HK) and a response regulator (RR). Contents within the external environment are sensed by and thereby activate HK, which in turn, phosphorylates RR resulting in the activation of an effector domain that produces the desired response for the present environmental condition [35]. The basic chemistry for a two-component activation and deactivation can be better visualized in Table 2. These systems allow bacteria to respond effectively

to the harsh host environments, including nitrogen and phosphate limitation, sugar transport and osmolarity by regulating effector proteins. For example, PhoP and PhoQ proteins express genes (*i.e.*, *phoP*, *phoQ*, and *pagC*) required for bacterial virulence and intracellular survival [24]. Mutant strains with defective *phoP* are less successful at surviving within macrophages and displayed increased sensitivity to small cationic peptides known as defensins [24].

### *Fimbriae*

Fimbriae are also significant elements for virulence through the initial bacterial invasion of the intestinal tract by mediating adhesion to the epithelial cells of the intestine [34]. Specifically *Salmonella* fimbriae adhere the bacteria to the M-cells of the intestine [4]. There have been 13 predicted fimbrial loci determined for *Salmonella* species, one of which is FimH that has specifically been shown to assist in the uptake of bacteria into murine dendritic cells independent of secretion systems [15]. These filamentous structures, also referred to as pili, are located on the outer membrane of *Salmonella* and are typically 2 to 8 nm wide and 0.5 to 10  $\mu\text{m}$  long [38]. Their primary components are fimbrins that are helically arranged repeating proteins.

### *Flagella*

As a virulence factor, flagella is most important for the initial stages of pathogenicity through the facilitation of host cell invasion by allowing *Salmonella* to swim to target cells within the host. It is also considered to be essential for prolonging of the infection. Flagella are long helical filaments attached to rotary motors integrated into the bacterial membrane and are designed for swimming or swarming [30].

Structurally, flagella require over 50 genes to synthesize the basal body, hook, and a filament, all of which make up its composition [37]. The filament of flagella is comprised of a protein called flagellin and this protein often recognized by host immune system as pathogen-associated molecular patterns (PAMPs) thereby activating the expression of proinflammatory cytokines [30, 41].

## HOST SPECIFICITY

Each host provides a unique environment of varying chemical and physical properties in which pathogens must persist by sustaining themselves throughout differing temperature, pH, osmolarity, and nutrient availability on top of evading host immune responses [25]. *Salmonella enterica* serovars can be identified as generalists, host-restricted, or host-specific. *S. Typhimurium* and *S. Enteritidis* are considered generalists because they have a broad range of hosts they can infect but the effects of infection vary from host to host. Although host-restricted species, such as *S. Dublin*, typically perpetuate disease in one particular host, it is capable of causing disease in other animals under certain circumstances. On the other hand, host-specific strains can only induce disease in one specific host. An example of this is *S. Typhi* because it only causes typhoid fever in humans. Host-restricted and host-specific strains are often associated with systemic infection in a particular host [27]. As stated, the pathogenicity of each strain can vary with their degree of host specificity. Paulin and colleagues (2002) determined that *Salmonella* colonization of calf intestines is affected by two

elements: (1) initial intestinal invasion and (2) subsequent bacterial persistence and growth, both of which are highly contingent on the degree of host specificity.

The initial surface contact between *Salmonella* and the host is the intestinal mucosa of the gut. This interaction can be delineated into three stages: (1) arrival and contact with intestinal mucosa, (2) penetration of or trapping by the mucus gel and (3) adhesion to epithelial cells [11]. The ability to bind to and colonize the intestinal mucosa along with other surfaces of target cells is essential to determining the host specificity of the strain. Not only is host specificity derived from the pathogen's ability to adhere to the target surfaces but surviving within host macrophages while counterbalancing host responses can determine whether the bacterium is a generalist, host-restricted, or host-specific.

#### *Host Response*

The immune system of the host is made up of multiple cell types that may contribute to the nuances of host specificity. For example, the immune system is equipped to detect LPS and lipoproteins of gram-negative bacteria. Once the pathogens are recognized, host immune factors typically function to successfully abolish bacterial infection. However, during instances where the pathogen is better equipped to survive, the immune system is still able to recognize the presence of pathogens thereby producing immune responses that show signs of the disease persisting. Host environment also contains antimicrobial peptides that are small, amphipathic molecules located on the epithelial surfaces inside phagocytes, which also colocalizes with NADPH-dependent oxidase that catalyzes the production of ROS [25]. Not only is



the host capable of producing ROS but also hinders foreign invasion through the presence of nitric oxide and other reactive nitrogen compounds that can be found within macrophages. *Salmonella* can combat these macrophage effects through the production of enzymes like superoxide dismutase.

#### *Macrophage survival*

Macrophages are phagocytic monocytes that can be found in almost all tissue types and are responsible for tissue homeostasis and immune response [22]. There have been conflicting views on the role of macrophages on host specificity due to discrepancies between *in vivo* and *in vitro* assays, which may be explained by the increased expression of cytokine factors. A study conducted by Xu and colleagues (2009) demonstrated that there was an increased expression of IL-6, IL-1 $\alpha$ , and TNF $\alpha$  during *S. Typhimurium* infection when compared to *S. Typhi* infection both of which also differ on uptake into macrophages, whereby *S. Typhi* is identified and cleared more quickly. Regardless of the controversy, surviving within macrophages is essential to *Salmonella* pathogenicity. There are two systems that play a role in macrophage survival: (1) PhoPQ, a two-component regulatory system and (2) SPI-2 T3SS [10].

Once the pathogen is ingested by the macrophage, the formation of a spacious phagosome that develops into SCV occurs as the macrophage begins to fuse with a lysosome. The lysosome then serves to break down the pathogen with acid hydrolases [6]. Recent studies have demonstrated that *Salmonella* will often multiply and divide within the SCV, which in turn divides the SCV resulting in one vacuole typically containing only one bacterium [6, 18]. Eswarrappa and colleagues (2010)

demonstrated that lysosomes are not produced in conjunction with the increasing SCVs but instead are actively reduced in number. SCV division causes a redistribution of components required for lysosomal biogenesis, such as vacuolar-type ATPase, LAMP1, LAMP2, cathepsin D, and acid phosphatase [6, 18]. Figure 2 depicts a probable hypothesis of how this may occur. The limited lysosomes will target SCVs containing clusters of bacteria; therefore, the number of bacteria found within each SCV plays a role in *Salmonella* host survival and cell proliferation.

Not only must the strain thwart lysosomal effects but it must also withstand ROS, reactive nitrogen species, antimicrobial peptides and adaptive host immune responses [12]. Effector proteins released from SPI-2 T3SS combat these effects. SPI-2 virulence genes are required for macrophage survival, intracellular proliferation and systemic infection [12]. Many of these virulence genes are regulated by two-component systems, such as SpiR/SsrB that can be found encoded into the SPI-2 pathogenicity island [8]. SsrB protein is required for the expression of SPI-2 T3SS and its effectors [8]. As stated previously, the PhoP/PhoQ two-component system is also essential for macrophage intracellular survival as demonstrated through the inability of mutant strains to proliferate. PhoP triggers the induction of SPI-2 in the presence of low magnesium levels and low pH such as that which can be found within macrophages [8].

#### NEUROPATHOGENIC STRAIN OF *SSTP*

In 2002, a neuropathogenic strain of *Sstp*, which will be referred as *Sstp*NPG from hereon, was first isolated from a 4-month old Holstein calf in Wisconsin that

displayed signs of diarrhea, head tilt, ataxia, auditory hyper-responsiveness, and partial blindness [23]. Field observations revealed a moderate morbidity rate of 40% on calves but a low mortality rate (0%). McCuddin and colleagues (2008) determined that the strain has a low infection rate due to *Sstp*NPG being recovered only from 1 of 12 calves. Standard hematoxylin and eosin (H & E) histopathology of the brain showed no significant signs of disease or inflammation and only one CFU from each 10 g of brain tissue was observed [23].

## PURPOSE

NTS encephalopathy is a rare occurrence due to the fact that systemic infection is not as common among these strains and even fewer bacterial pathogens are capable of crossing the BBB. Interestingly, *Sstp*NPG is capable of inducing encephalopathy in stressed calves. Calves infected by *Sstp*NPG and stressed from transportation and/or co-mingling often show signs of ear fluttering and seizures. This alludes to a relationship between host neuroendocrine hormones and the traversal of the BBB. Once inside the brain, little is known on the mechanism of infection that would produce these signs of neurological deficiencies. A better understanding of this may lead to pharmacological developments and/or ways to abrogate the disease. In order to gain knowledge on *Sstp*NPG and its relationship with calves the following were ascertained: (1) host condition specificity of the strain (2) genes involved in the traversal of BBB and the relationship it has with neuroendocrine hormones, (3) abrogation of symptoms

through antibiotics, and (4) identification of factors involved in perpetuating encephalopathy.

## REFERENCES

1. Bell RL, González-Escalona N, Stones R, and Brown EW. 2011. Phylogenetic evaluation of the 'Typhimurium' complex of *Salmonella* strains using a seven-gene multi-locus sequence analysis. *Infection, Genetics, and Evolution*, 11(1): 83-91. DOI:10.1016/j.meegid.2010.10.005.
2. Beyer W, Mukendi FM, Kimmig P, and Böhm R. 1998. Suitability of repetitive-DNA-sequence-based PCR fingerprinting for characterizing epidemic isolates of *Salmonella enterica* serovar Saintpaul. *Journal of Clinical Microbiology*, 36(6): 1549-1554.
3. Bischofberger M and van der Goot FG. 2008. Exotoxin secretion: getting out to find the way in. *Cell Host & Microbe Previews*, 3: 7-8. DOI:10.1016/j.chom.2007.12.003.
4. Edwards RA, Schifferli DM, and Maloy SR. 2000. A role for *Salmonella* fimbriae in intraperitoneal infections. *Proceedings of the National Academy of Sciences*, 97(3): 1258-1262.
5. Ernst RK, Guina T, and Miller SI. 1999. How intracellular bacteria survive: surface modifications that promote resistance to host innate immune responses. *Journal of Infectious Diseases*, 179(Suppl 2): S326-30. DOI: 10.1086/513850.
6. Eswarappa SM, Negi VD, Chakraborty S, Sagar BKC, and Chakravorty D. 2010. Division of the *Salmonella*-containing vacuole and depletion of acidic lysosomes in *Salmonella*-infected host cells are novel strategies of *Salmonella enterica* to avoid lysosomes. *Infection and Immunity*, 78(1): 68-79. DOI:10.1128/IAI.00668-09.
7. Eswarappa SM, Janice J, Nagarajan AG, Balasundaram SV, Karnam G, Dixit NM, and Chakravorty D. 2008. Differentially evolved genes of *Salmonella* pathogenicity islands: insights into the mechanism of host specificity in *Salmonella*. *Public Library of Science One*, 3(12): e3829. DOI:10.1371/journal.pone.0003829.

8. Fass E and Groisman EA. 2009. Control of *Salmonella* pathogenicity island-2 gene expression. *Current Opinions of Microbiology*, 12(2): 199-204. DOI:10.1016/j.mib.2009.01.004.
9. Foley SL and Lynne AM. 2008. Food animal-associated *Salmonella* challenges: Pathogenicity and antimicrobial resistance. *Journal of Animal Science*, 86: E173-E187. DOI: 10.2527/jas.2007-0447.
10. Forest CG, Ferraro E, Sabbagh SC, and Daigle F. 2010. Intracellular survival of *Salmonella enterica* serovar Typhi in human macrophages is independent of *Salmonella* pathogenicity island (SPI)-2. *Microbiology*, 156: 3689-3698. DOI:10.1099/mic.0.041624-0.
11. Frost AJ, Bland AP, and Wallis TS. 1997. The early dynamic response of the calf ileal epithelium to *Salmonella typhimurium*. *Veterinary Pathology*, 34: 369-386. DOI:10.1177/030098589703400501.
12. Ge S, Danino V, He Q, Hinton JCD, and Granfors K. 2010. Microarray analysis of response of *Salmonella* during infection of HLA-B27- transfected human macrophage-like U937 cells. *BioMed Central Genomics*, 11: 456-459. DOI:10.1186/1471-2164-11-456.
13. Hansen-Wester I and Hensel M. 2001. *Salmonella* pathogenicity islands encoding type III secretion systems. *Microbes and Infection*, 3: 549-559. DOI:10.1016/S1286-4579(01)01411-3.
14. Huehn S and Malorny B. Caugant DA (ed). 2009. Chapter 19: DNA microarray for molecular epidemiology of *Salmonella*. *Molecular Epidemiology of Microorganisms, Methods in Molecular Biology*, 551: 249-285. DOI: 10.1007/978-1-60327-999-4\_19.
15. Ibarra JA and Steele-Mortimer O. 2009. *Salmonella* – the ultimate insider. *Salmonella* virulence factors that modulate intracellular survival. *Cellular Microbiology*, 11(11): 1579-1586. DOI:10.1111/j.1462-5822.2009.01368.
16. Khan SA, Everest P, Servos S, Foxwell N, Zähringer U, Brade H, Rietschel ETh, Dougan G, Charles IG, and Maskell DJ. 1998. A lethal role for lipid A in *Salmonella* infections. *Molecular Microbiology*, 29(2): 571-579. DOI: 10.1046/j.1365-2958.1998.00952.x.
17. Kraehenbuhl J-P and Neutra MR. 2000. Epithelial M cells: differentiation and function. *Annual Review of Cell and Developmental Biology*, 16: 301-332. DOI:10.1146/annurev.cellbio.16.1.301.

18. Lahiri A, Lahiri A, Iyer N, Das P, and Chakravortty D. 2010. Visiting the cell biology of *Salmonella* infection. *Microbes and Infection*, 12(11): 809-818. DOI:10.1016/j.micinf.2010.05.010.
19. Liao C-H, Cooke PH, and Niemira BA. 2010. Localization, growth, and inactivation of *Salmonella* Saintpaul on jalapeño peppers. *Journal of Food Science*, 75(6): M377-M382. DOI: 10.1111/j.1750-3841.2010.01667.x.
20. Libby SJ, Adams LG, Ficht TA, Allen C, Whitford HA, Buchmeier NA, Bossie S, and Guiney DG. 1997. The spv genes on the *Salmonella dublin* virulence plasmid are required for severe enteritis and systemic infection in the natural host. *Infection and Immunity*, 65: 1786-1792.
21. Leung KY, Siame BA, Snowball H and Mok Y. 2010. Type IV secretion regulation: crosstalk and intracellular communication. *Current Opinion in Microbiology*, 14: 1-7. DOI 10.1016/j.mib.2010.09.017.
22. Lucas AD and Greaves DR. 2001. Atherosclerosis: role of chemokines and macrophages. *Expert Review of Molecular Medicine*, 3(25): 1-18. DOI:10.1017/S1462399401003696.
23. McCuddin ZP, Carlson SA, and Sharma VK. 2008. Experimental reproduction of bovine *Salmonella* encephalopathy using a norepinephrine-based stress model. *The Veterinary Journal*, 175: 82-88. DOI:10.1016/j.tvjl.2006.12.014. DOI:10.1016/j.tvjl.2006.12.014.
24. Miller SI, Kukral AM, and Mekalanos JJ. 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. *Proceedings of the National Academy of Sciences*, 86: 5054-5058.
25. Ohl ME and Miller SI. 2001. *Salmonella*: a model for bacterial pathogenesis. *Annual review of Medicine*, 52: 259-274. DOI: 10.1146/annurev.med.52.1.259.
26. Paulin SM, Watson PR, Benmore AR, Stevens MP, Jones PW, Villarreal-Ramos B, and Wallis TS. 2002. Analysis of *Salmonella enterica* serotype-host specificity in calves: avirulence of *S. enterica* serotype Gallinarum correlates with bacterial dissemination from mesenteric lymph nodes and persistence *in vivo*. *Infection and Immunity*, 70(12): 6788-6797. DOI: 10.1128/IAI.70.12.6788-6797.2002.
27. Pullinger GD, van Diemen PM, Dziva F, and Stevens MP. 2010. Role of two-component sensory systems of *Salmonella enterica* serovar Dublin in pathogenesis of systemic salmonellosis in cattle. *Microbiology*, 156: 3108-3122. DOI:10.1099/mic.0.041830-0.

28. Raffatellu M, Wilson RP, Winter SE, Bäumler AJ. 2008. Clinical pathogenesis of typhoid fever. *Journal of Infection in Developing Countries*, 2(4): 260-266.
29. Rotger R and Casadesus J, 1999. The virulence plasmids of *Salmonella*. *International Microbiology*, 2: 177-184.
30. Saini S, Slauch JM, Aldridge PD, and Rao CV. 2010. Role of cross talk in regulating the dynamic expression of the flagellar *Salmonella* pathogenicity island 1 and type 1 fimbrial genes. *Journal of Bacteriology*, 192(21): 5767-5777. DOI:10.1128/JB.00624-10.
31. Santos RL, Zhang S, Tsois RM, Bäumler AJ, and Adams LG. 2002. Morphologic and molecular characterization of *Salmonella typhimurium* infection in neonatal calves. *Veterinary Pathology*, 39: 200-215. DOI: 10.1354/vp.39-2-200.
32. Santos RL, Zhang S, Tsois RM, Kingsley RA, Adams LG, and Bäumler AJ. 2001. Animal models of *Salmonella* infections: enteritis versus typhoid fever. *Microbes and Infection*, 3: 1355-1344. DOI:10.1016/S1286-4579(01)01495-2.
33. Spanò S, Ugalde JE, and Galán JE. 2008. Delivery of *Salmonella* Typhi exotoxin from a host intracellular compartment. *Cell Host & Microbe*, 3: 30-38. DOI:10.1016/j.chom.2007.11.001.
34. Stevens MP, Humphrey TJ, and Maskell DJ. 2009. Molecular insights into farm animal and zoonotic *Salmonella* infections. *Philosophical Transactions of the Royal Society*, 364: 2709-2723. DOI:10.1098/rstb.2009.0094.
35. Stock AM, Robinson VL, and Goudreau PN. 2000. Two-component signal transduction. *Annual Review of Biochemistry*, 69: 183-215. DOI:10.1146/annurev.biochem.69.1.183
36. Suárez M and Rüssmann H. 1998. Molecular mechanisms of *Salmonella* invasion: the type III secretion system of the pathogenicity island 1. *International Microbiology*, 1: 197-204.
37. Uchiya K, Sugita A, and Nikai T. 2009. Involvement of SPI-2-encoded SpiC in flagellum synthesis in *Salmonella enterica* serovar Typhimurium. *BioMed Central Microbiology*, 9: 179. DOI:10.1186/1471-2180-9-179.
38. van Asten AJAM and van Dijk JE. 2005. Distribution of “classic” virulence factors among *Salmonella* spp. *FEMS Immunology and Medical Microbiology*, 44: 251-259. DOI:10.1016/j.femsim.2005.02.002.

39. Xi M, Zheng J, Zhao S, Brown EW and Meng J. 2008. An enhanced discriminatory pulsed-field gel electrophoresis scheme for subtyping *Salmonella* serotypes Heidelberg, Kentucky, SaintPaul, and Hadar. *Journal of Food Protection*, 71(10): 2067-2072.
40. Xu T, Maloy S, and McGuire KL. 2009. Macrophage influence *Salmonella* host-specificity in vivo. *Microbial Pathogenesis*, 47: 212-222. DOI:10.1016/j.micpath.2009.07.004.
41. Zipfel C, Robatzek S, Navarrow L, Oakeley EJ, Jones JDG, Felix G, and Boller T. 2004. Bacterial disease resistance in Arabidopsis through flagellin perception. *Nature*, 428: 764-767. DOI:10.1038/nature02485.



Table 1. Comparison between bacterial endotoxins and exotoxins

PROPERTY	ENDOTOXIN	EXOTOXIN
Molecular component	Lipopolysaccharide	Protein
Location	Outer membrane	Diffusible element
Potency	Low (> 100µg)	High (1 µg)
Specificity	Low	High
Enzymatic activity	No	Yes

Table 2. Basic chemistry of a two-component phosphoryl transfer signal transduction pathway (as seen in Stock *et al.* 2000)

TYPE OF PHOSPHORYL TRANSFER	SIGNAL TRANSDUCTION PATHWAY
Autophosphorylation	$\text{HK-His} + \text{ATP} \rightleftharpoons \text{HK-His}\sim\text{P} + \text{ADP}$
Phosphotransfer	$\text{HK-His}\sim\text{P} + \text{RR-Asp} \rightleftharpoons \text{HK-His} + \text{RR-Asp}\sim\text{P}$
Dephosphorylation	$\text{RR-Asp}\sim\text{P} + \text{H}_2\text{O} \rightleftharpoons \text{RR-Asp} + \text{P}_i$

Autophosphorylation occurs on the histidine side chain of the HK, indicated by HK-His. HK then transfers the phosphoryl group,  $\sim\text{P}$ , to the aspartate side chain of RR, designated with RR-Asp. Dephosphorylation of RR occurs when the phosphoryl group is transferred to water,  $\text{H}_2\text{O}$ .

Figure 1. *S. Typhimurium* gaining entrance into a Hep-2 cell via modification of host actin cytoskeleton and perpetuation of macropinocytosis mediated through membrane ruffles (as seen in Ohl and Miller 2001).

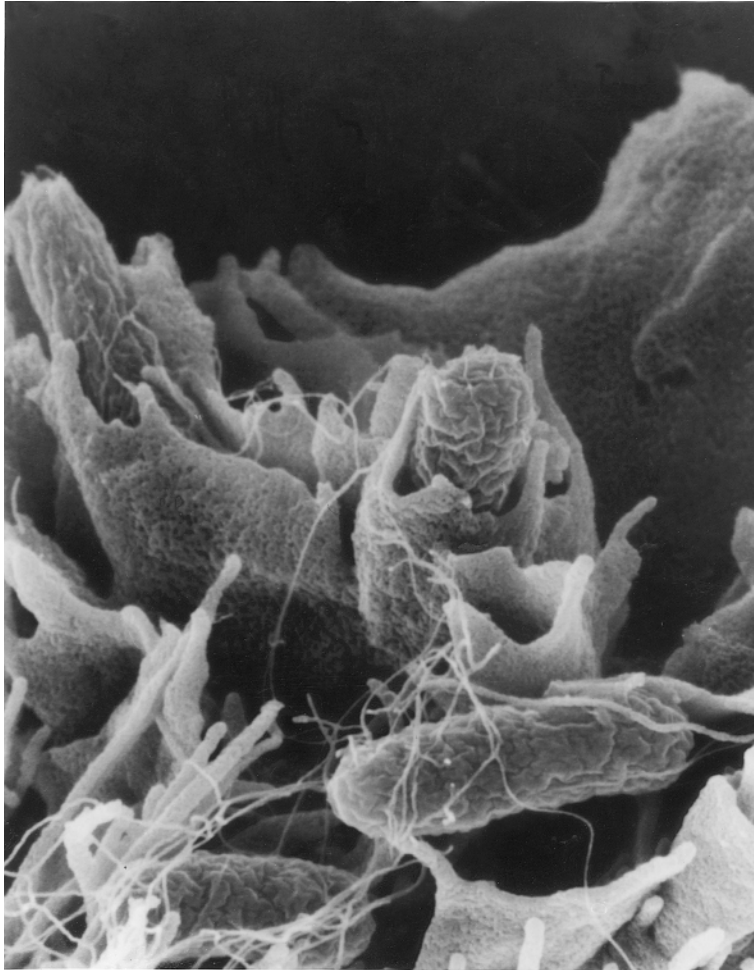
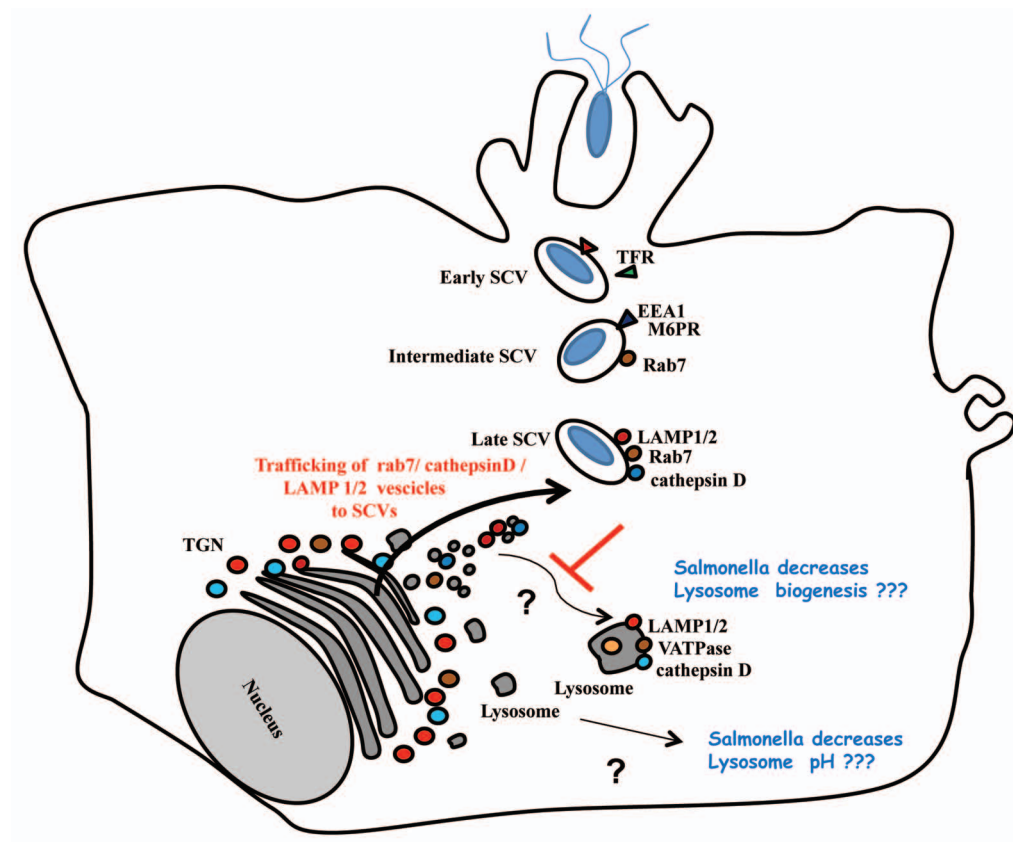


Figure 2. Possible trafficking of lysosomes to SCV (as seen in Lahiri *et al.* 2010)



## **CHAPTER 2. EVALUATION OF THE PATHOGENICITY AND VIRULENCE OF THREE STRAINS OF SALMONELLA ORGANISMS IN CALVES AND PIGS**

Modification of a paper published in  
*American Journal of Veterinary Research*

Nalee Xiong, Matt T Brewer, Tim A Day, Michael J Kimber,  
Alison E Barnhill, and Steve A Carlson

### **ABSTRACT**

The objective of this study was to assess in pigs the pathogenicity and virulence of *Salmonella enterica* serovar Saintpaul, which is capable of causing nontyphoidal *Salmonella* encephalopathy (NTSE) in cattle. By experimentally infecting Holstein calves and pigs, clinical manifestations of pigs were compared with those observed in cattle. *Salmonella*-mediated encephalopathy and multisystemic cytopathicity did not appear to be a relevant disease in swine.

### **INTRODUCTION**

During the past 10 years, a unique type of salmonellosis in cattle has been identified and characterized by our laboratory group. This pathotype involved 3 strains of *Salmonella* capable of causing neurologic disease in cattle recently exposed to stressful situations such as transportation or co-mingling. These strains included *S. enterica* serotypes Saintpaul, Montevideo, and Enteritidis isolated from calves in Minnesota and Wisconsin [4]. Affected calves had signs of moderate to severe signs of neurologic disease ranging from excessive ear fluttering to seizures, and some calves

had permanent neurologic deficits. The neurologic effects of these strains were reproduced in a laboratory setting using a norepinephrine-based stress-induction technique [4]. These outbreaks of *Salmonella* encephalopathy are now extremely rare, but the potential for zoonotic transmission and the dramatic nature of the disease warrant further investigation in other food-producing animals.

Because of anecdotal reports suggesting unusual aspects of extraintestinal salmonellosis in pigs, the study reported here also investigated the potential for *Salmonella*- associated neuropathogenicity in pigs. For this neuropathogenicity study, subjects were inoculated with neuropathogenic *Salmonella* Saintpaul (*Sstp*NPG) and then monitored for stress-mediated neurologic disease and evidence of *Salmonella* organisms in various tissues.

## MATERIALS AND METHODS

### *Animals, bacterial strain and preparation*

The *in vivo* experiment involved the use of 1-to 2-week-old male Holstein calves and 10-day old mixed-breed pigs of both sexes. Animal experiments were approved by the Iowa State University Institutional Animal Care and Use Committee.

*Sstp*NPG was chosen as a representative neuropathogenic strain. *Sstp*SARB, which is unable to induce signs of neurologic disease, was also used to inoculate a control group. Bacteria were stored in cryopreservation tubes containing 50% glycerol and 50% Lennox L broth at -80°C and were grown in or on Lennox L broth or culture agar prior to use.

*In vivo infection-stress experiments involving neuropathogenic strains*

Animals were challenge-exposed via oral administration of  $4.5 \times 10^6$  CFUs of *SstpNPG*/kg; these *Salmonella* organisms were grown aerobically for 16 hours at 37°C. For each strain, 3 pigs and 3 calves received daily doses of norepinephrine (45 µg/kg, IM) starting on the day of inoculation and continuing until the animals were euthanatized. As a neurohormonal control treatment, 3 calves and 3 pigs were challenge-exposed by oral administration of *SstpNPG* and administered a placebo (volume of saline [0.9% NaCl] solution identical to the volume of norepinephrine, IM). Additionally, 3 calves and 3 pigs were challenge-exposed by oral administration of *SstpNPG* and administered daily doses of dexamethasone (0.1 mg/kg, IM). The control strain, which was administered to 3 calves and 3 pigs, was *SstpSARB*, which is not capable of causing neurologic disease in cattle [3].

Animals were monitored for neurologic disease (seizures or clinical signs such as excessive ear fluttering, ataxia, opisthotonus, and proprioceptive placing deficits). When signs of neurologic disease were observed (typically 4 to 9 days after inoculation), affected animals were immediately euthanatized. An animal with neurologic disease was defined as an animal that had convulsions or that had at least 2 of the aforementioned 4 clinical signs of neurologic disease. Animals that did not have signs of neurologic disease were euthanatized 12 to 14 days after inoculation (*i.e.*, at least 3 or 4 days after animals with signs of neurologic disease were euthanatized). Animals were euthanatized with xylazine and pentobarbital, as described previously. Samples of the intestines, blood, spleen, and brain were collected from each animal.

*Bacteriologic-based detection of Salmonella spp*

For qualitative assessment of *Salmonella* spp, microbes were selectively cultured by inoculation of 3 to 5 g of each sample into 100 mL of GN Hajna broth and incubation overnight at 37 °C in aerobic conditions. Then, 100 µL of the inoculum was transferred into 5 mL of Rappaport-Vassiliadis R10 broth, which was also incubated overnight at 37°C in aerobic conditions. Cultures were then transferred to plates containing BGS agar; plates were incubated overnight at 37°C. Individual colonies recovered from selective plates were grown overnight in Lennox L broth and identified by use of a *Salmonella*-specific PCR assay targeting the sipB-sipC junction [2]. All media included antimicrobials specific to the antibiogram of the individual isolate.

For quantitation of *Salmonella* organisms in blood and spleen tissues as an estimate of systemic pathogen load and as an indirect correlate of clinical signs, samples were directly inoculated on plates containing BGS agar. For blood samples, 100 µL was inoculated onto each of 10 separate plates. For spleen samples, 50 to 75 g of sample was homogenized, filtered with cheesecloth, and then inoculated onto 10 plates containing BGS agar (*i.e.*, approx 3 to 5 g of sample/plate). Colonies were enumerated the following day. Because these tissues can harbor similar numbers of bacteria at early time points in the infectious process, CFU data for spleen samples were pooled with counts from blood samples to provide the final data of each time course experiment. The identity of *Salmonella* strains was confirmed by use of antisera-agglutination-based serogrouping [1] in addition to confirmation of the original antibiogram for each isolate.



## RESULTS

In vivo experiments were conducted to determine whether the *SstpNPG* strain could lead to neurologic disease in pigs, similar to that observed in stressed calves [3]. *SstpNPG* was inoculated into young calves and pigs, and these animals were also administered consecutive daily doses of norepinephrine or dexamethasone to mimic stress. As a control sample, animals were inoculated with *SstpSARB*, which is not capable of eliciting signs of neurologic disease in calves [3].

Pigs did not have signs of clinical disease, and *Salmonella* organisms could only be recovered from the intestines (Table 1). In contrast, calves had signs of neurologic disease when inoculated with *SstpNPG* and administered norepinephrine or dexamethasone. *Salmonella* organisms were isolated from the intestines, blood, spleen, and brain of calves with signs of neurologic disease. Non-neuropathogenic *SstpSARB* was only recovered from the intestines of calves, similar to results in another study [3].

## DISCUSSION

Several unique strains of *Salmonella* spp have unusual virulence and pathogenicity properties in calves. However, these effects have not been confirmed clinically in pigs. Neuropathogenicity have been associated with specific strains of *Salmonella* spp under certain conditions. Stress has been implicated in the etiology of the encephalopathies related to the neuropathogenic *Salmonella* spp. *SstpNPG* caused neurologic disease in cattle, but similar effects were not observed in pigs. This finding is not altogether surprising because the *Salmonella*-associated neuropathogenicity

appears to be dependent on neurohormonal factors in cattle that may be divergent from those in swine. Overall, *Salmonella* strains are typically more virulent in cattle.

## REFERENCES

1. Carlson SA, Stoffregen WC, Bolin SR. 2002. Abomasitis due to multiple antibiotic resistant *Salmonella enterica* serotype Typhimurium phage DT104. *Veterinary Microbiology*, 85(3): 233-240. DOI:10.1016/S0378-1135(01)00508-9.
2. Carlson SA, Bolton LF, Briggs CE, Hurd HS, Sharma VK, Fedorka-Cray PJ, Jones BD. 1999. Detection of multiresistant *Salmonella* typhimurium DT104 using multiplex and fluorogenic PCR. *Molecular and Cellular Probes*, 13(3): 213-222. DOI:10.1006/mcpr.1999.0240.
3. McCuddin ZP, Carlson SA, and Sharma VK. 2008. Experimental reproduction of bovine *Salmonella* encephalopathy using a norepinephrine-based stress model. *The Veterinary Journal*, 175: 82-88. DOI:10.1016/j.tvjl.2006.12.014.
4. Rasmussen MA, Carlson SA, Franklin SK, McCuddin ZP, Wu MT, and Sharma VK. 2005. Exposure to rumen protozoa leads to enhancement of pathogenicity of and invasion by multiple-antibiotic resistant *Salmonella enterica* bearing SGI1. *Infection and Immunity*, 73(8): 4668-4675. DOI:10.1128/IAI.73.8.4668-4675.2005.

Table 1. Qualitative assessment of *Salmonella* organisms recovered from male Holstein calves and male and female mixed-breed pigs inoculated with *S. enterica* serotype SaintpaulNPG or non-neuropathogenic *Salmonella* SaintpaulSARB and injected with norepinephrine or dexamethasone to simulate stress.

Strain	Treatment	Cattle				Swine			
		Tissue source			Neurologic disease*	Tissue source			Neurologic disease*
		In	Bl-S	Br		In	Bl-S	Br	
SstpNPG	Saline**	A	NI	NI	A	A	NI	NI	NI
	Norepinephrine	A	A	A	A	A	NI	NI	NI
	Dexamethasone	A	B	B	B	A	NI	NI	NI
SstpSARB	Norepinephrine	A	NI	NI	NI	A	NI	NI	NI

\* Neurologic disease was defined as animals that had convulsions or that had at least 2 of 4 clinical signs of neurologic disease (excessive ear fluttering, ataxia, opisthotonus, and proprioceptive placing deficits)

\*\* 0.9% NaCl solution

A = Isolated or observed in all 3 animals

B = Isolated or observed in 2 of 3 animals

Bl-S = Blood and spleen combined

Br = Brain

In = Intestines

NI = Not isolated or identified

### **CHAPTER 3. EXPRESSION OF A COLLAGENASE THAT ENABLES BLOOD-BRAIN BARRIER PENETRATION FOR *SALMONELLA* IMPLICATED IN BOVINE ENCEPHALOPATHIES**

Modification of a paper in submission to  
*Microbial Pathogenesis*

Nalee Xiong, Matthew T. Brewer, Kristi L. Anderson, Katharine E. Weeks,  
and Steve A. Carlson

#### **ABSTRACT**

Recent studies identified strains of *Salmonella* that induce encephalopathies in calves exposed to stressful situations. In order to cause neurologic signs (such as ataxia, head tilt, and partial blindness), the strain must be able to cross the blood-brain barrier (BBB). One possible way is through the breakdown of tight junctions, which regulate the permeability of the BBB and can be weakened by enzymes such as collagenases. *Salmonella* and other Gram-negative bacteria contain a collagenase gene (*clg*) that is silenced *in vitro* but inducibly expressed *in vivo*. We hypothesized that the neuropathic strains of *Salmonella* express *clg* in response to neuroendocrine factors in the host and that the expressed collagenase perturbs the BBB allowing for CNS invasion by *Salmonella*. Our *in vitro* results revealed that *clg* is derepressed in serum obtained from stressed cattle. Derepression is relegated to the neuropathic *Salmonella* strains. *In vivo* studies indicated that *clg* expression is required for neuropathogenicity and that pharmacologic maintenance of the BBB prevents both the *Salmonella* invasion into the brain and the resulting neurologic signs. These studies identify a host-induced *Salmonella* collagenase that facilitates neuropathogenicity at the level of the BBB.

## INTRODUCTION

*Salmonella* infections are a continuous concern for human medicine, veterinary medicine, and food safety. Salmonellosis is based on the ability of the bacterium to secrete toxins, invade host cells, and survive within macrophages [6, 13]. Systemic salmonellosis is a common outcome and neurologic disease is a rare yet devastating sequela for typhoidal and paratyphoidal serotypes that are adapted and/or restricted to certain hosts [4]. Non-typhoidal encephalopathies are very rare but we identified three non-typhoidal isolates capable of causing encephalopathies in stressed calves. A neuropathogenic isolate of *Salmonella enterica* serovar Saintpaul (*Sstp*NPG) was isolated from a four-month old Holstein calf exhibiting signs of encephalopathy in 2002 [9] and this isolate is the paradigm for the studies described herein.

Our previous studies [9] revealed that *Sstp*NPG is present in the brains of calves displaying neurologic signs suggesting that this isolate traverses the blood-brain barrier (BBB). This phenomenon occurred in the absence of overt CNS inflammation that typically compromises the BBB leading to typhoidal encephalopathies. Therefore, it appears that *Sstp*NPG must circumvent the BBB in an alternative manner.

The BBB is comprised of tight junctions and adherens junctions between blood vessels and a basement membrane composed of type IV collagen, laminin, proteoglycan, glycoproteins, and astroglial end feet [1, 5]. Depletion of tight junctions and degradation of the basement membrane can lead to a paracellular route through the BBB and into the brain. Therefore, a collagenase may be able to mediate this process.

Our previous studies revealed that a *Salmonella* collagenase causes atypical salmonellosis in veal calves reared in confined spaces, *i.e.*, stressed animals [13]. Since *Sstp*NPG only induces neuropathogenicity in stressed calves, we set out to determine if *clg* plays a role in *Sstp*NPG translocation across the BBB and whether the derepression of *clg* results from specific neuroendocrine hormone interactions.

## MATERIALS AND METHODS

### *Bacterial strains*

*Sstp*NPG and *Sstp*SARB were as described previously [9]. *Sstp*NPG was also transformed with a pCRXL plasmid bearing *clg* (pAs-Clg) oriented in the antisense direction, serving to knock down the gene expression in the recombinant isostrain [13]. HB101, a laboratory strain of *E. coli* (Invitrogen), was transformed with a plasmid that constitutively expresses collagenase (pClg) from the *lac* promoter on pCRXL (Invitrogen) [13].

### *In vitro experiments assessing clg derepression*

A recently transported Angus steer provided the “stressed” serum while an adult dairy cow provided the “unstressed” serum. Serum and LB broth were mixed 1:1 and  $10^8$  CFUs of bacteria were added to the mixture. To test the effects of neuroendocrine hormones, norepinephrine (50 µg/mL; Sigma) and dexamethasone (100 µg/mL; Sigma) were individually added to 2 mL of LB broth containing  $10^8$  CFUs of bacteria. HB101-pClg was grown in 2 mL of LB broth with no treatment and served as the positive control for *clg* expression. All incubations were performed aerobically at 37°C

overnight. *Sstp*NPG was grown in the presence of 8 µg/ml of ceftiofur since this isolate expresses an extended-spectrum beta-lactamase. *Sstp*NPG/pAs-Clg and HB101-pClg were grown in the presence of 32 µg/ml of kanamycin since the pAs-Clg and pClg plasmids contain a kanamycin resistance marker.

Following the incubation, RNA was isolated from the bacteria grown using the RNeasy kit (Invitrogen) in order to determine the expression of collagenase via RT-PCR. The OneStep RT-PCR kit was used with the following oligonucleotide primers: ClgF (5'-AGGACTGTATGCGCGCCAGCAAC-3') and ClgR (5'-GTCGACCTCGAGTTAAACGCCCTGCGCTT CGG-3'). Thermocycling was as follows: 55°C for 30 minutes, 94°C for 2 minutes, and then 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 68 degrees for 1 minute.

#### *In vivo experiments*

*In vivo* experiments involved Holstein calves 1 to 5 weeks of age (30 to 60 kg). All subjects were pharmacologically stressed through daily intramuscular injections of norepinephrine as per McCuddin *et al.* [9]. Eight calves were orally challenged with 1 mL inoculum containing 10<sup>9</sup> CFUs of *Sstp*NPG, which was grown aerobically overnight for 16 hours at 37°C. Four of these calves served as the control group and received no additional treatments while the other group of four calves received cilostazol (Sigma Chemicals; 1 mg/kg, IM, s.i.d.; [3]). An additional four calves received a strain of *Sstp*NPG in which *clg* is transiently knocked down via an antisense expression plasmid (pAs-Clg). The final group of four calves were inoculated with the *Sstp*SARB isostrain that does not elicit neurologic disease [9].

Indications of the nervous system being compromised were observed through behavioral aberrations such as head tilt, ataxia and other movement anomalies. Calves were euthanized immediately after showing signs of neurologic disease (3 to 6 days post-infection) and those that did not display encephalopathy were euthanized 8 to 10 days post-infection. Euthanasia was performed using xylazine (0.45 mg/kg, IM, Phoenix Laboratories) and pentobarbital (1.2 mg/kg, IV, Fort Dodge Laboratories). Animal experiments were approved by the Institutional Animal Care and Use Committee at Iowa State University (protocol 1-08-6508B).

#### *Qualitative Salmonella assessment*

Blood (10 mL) and brain (10 g) samples were collected for the qualitative isolation of *Salmonella*. Samples were enriched in a 50 mL falcon tube and grown statically overnight at 37°C in 10 mL of LB broth with no antibiotics. *Salmonella* colonies were selectively cultured and isolated on brilliant green agar plates containing 8 µg/mL of ceftiofur. Colonies were identified using *Salmonella*-specific PCR targeting the *sipB-sipC* junction [13].

## RESULTS

#### *Assessment of host neuroendocrine hormones on *clg* expression*

In order to determine if there is a relationship between host neuroendocrine hormones and *clg* derepression, both *Sstp*NPG and a SARB control *Sstp* strain [7] were grown in nutrient broth containing serum from stressed or unstressed cattle. Additionally, both *Sstp*NPG and the control *Sstp*SARB strain were grown in broth



containing either norepinephrine or dexamethasone. HB101-pClg, an *E. coli* that constitutively expresses recombinant *clg*, was grown in untreated broth as a positive control for *clg* expression. Each condition was conducted in 2 mL broth mixtures that were aerobically grown for 16 hours overnight at 37°C in a tabletop shaker. RNA was then isolated and used as the template to determine whether the *clg* was derepressed. As shown in Figure 1, RT-PCR assays indicated that *clg* was only transcribed in the presence of stressed calf serum. Neither norepinephrine nor dexamethasone was able to solely derepress gene expression.

*Repression of clg prevented SstpNPG from traversing the BBB*

In order to determine if there is a direct relationship between *clg* and the success of the strain at traversing the BBB, calves were pharmacologically stressed (using norepinephrine) and orally challenged with an isostrain of *SstpNPG* incapable of expressing *clg*. As shown in Table 1, repression of *clg* obviated neurologic disease and BBB penetration of *SstpNPG* that was recovered from the systemic circulation.

*Cilostazol prevents SstpNPG from traversing the BBB*

In order to determine if *SstpNPG* elicits changes in the BBB, calves were inoculated with *SstpNPG* and treated with norepinephrine and cilostazol. Cilostazol maintains the integrity of the BBB by increasing intracellular cAMP concentrations through the selective inhibition of phosphodiesterase III [7, 8]. As shown in Table 1, cilostazol prevented neurologic disease and BBB penetration of *SstpNPG*. As observed with repression of *clg* in the *clg*-knockdown isostrain, *SstpNPG* was recovered from the systemic circulation in cilostazol-treated calves but was not isolated from calf brains.

## DISCUSSION

Our *in vitro* experiments demonstrated that derepression of *clg* specifically involved host neuroendocrine hormone elements. Though our results indicate this derepression is not specifically due to norepinephrine or cortisol (represented by dexamethasone) alone, it is possible that both still play a role. Stress leads to the activation of the autonomic nervous system and hypothalamo-pituitary-adrenal (HPA) axis. There are a number of mediators aside from catecholamines and glucocorticoids that play a role in how the body reacts to stress, whereby each mediator has the ability to regulate the activity of other mediators in a network [10]. Many studies have determined a connection between host neuroendocrine stress hormones and increased bacterial virulence but little is known on its role in derepression of *clg*. Due to the complexity and little research conducted on this physiological process, further experiments need to be conducted to address the relationship between neuroendocrine hormones and the derepression of *clg*.

*In vivo* experiments demonstrated that *clg* was essential for inducing neuropathogenicity and repressing *clg* resulted in bacteria remaining in the systemic circulation. Maintaining the integrity of the BBB also prevented neurologic disease by sequestering the pathogen in the systemic circulation. These results suggest that the collagenase cleaves tight junctions allowing for bacterial penetration into the brain via a paracellular route.

## REFERENCES

1. Cardoso FL, Brites D, and Brito MA. 2010. Looking at the blood-brain barrier: molecular anatomy and possible investigation approaches. *Brain Research Reviews*, 64(2): 328-363. DOI:10.1016/j.brainresrev.2010.05.003.
2. Carlson SA, Bolton LF, Briggs CE, Hurd HS, Sharma VK, Fedorka-Cray PJ, Jones BD. 1999. Detection of multiresistant *Salmonella* typhimurium DT104 using multiplex and fluorogenic PCR. *Molecular and Cellular Probes*, 13(3): 213-222. DOI:10.1006/mcpr.1999.0240.
3. Choi J, Shin HK, Kim KY, Lee JH, and Hong KW. 2002. Neuroprotective effect of cilostazol against focal cerebral ischemia via antiapoptotic action in rats. *Journal of Pharmacology and Experimental Therapeutics*, 300(3): 787-793. DOI:10.1124/jpet.300.3.787.
4. Cohen JL, Bartlett JA, and Corey GR. 1987. Extra-intestinal manifestations of *Salmonella* infections. *Medicine*, 66(5): 349-387.
5. Hawkins R, O'Kane RL, Simpson IA, and Viña JR. 2006. Structure of the blood-brain barrier and its role in the transport of amino acids. *Journal of Nutrition*, 136(1 Suppl): 218S-226S.
6. House D, Bishop A, Parry C, Dougan G, and Wain J. 2001. Typhoid fever: pathogenesis and disease. *Current Opinion in Infectious Diseases*, 14(5): 573-578.
7. Kimura Y, Tani T, Kanbe T, and Watanabe K. 1985. Effect of cilostazol on platelet aggregation and experimental thrombosis. *Arzneimittelforschung*, 35(7A): 1144-1149.
8. Lee J, Kim KY, Lee YK, Park SY, Kim CD, Lee WS, Rhim BY, and Hong KW. 2004. Cilostazol prevents focal cerebral ischemic injury by enhancing casein kinase 2 phosphorylation and suppression of phosphatase and tensin homolog deleted from chromosome 10 phosphorylation in rats. *Journal of Pharmacology and Experimental Therapeutics*, 308(3): 896-903. DOI:10.1124/jpet.103.061853.
9. McCuddin ZP, Carlson SA, and Sharma VK. 2008. Experimental reproduction of bovine *Salmonella* encephalopathy using a norepinephrine-based stress model. *The Veterinary Journal*, 175: 82-88. DOI:10.1016/j.tvjl.2006.12.014.
10. McEwen BS. 2007. Physiology and neurobiology of stress and adaptation: central role of the brain. *Physiology Reviews*, 87(3): 873-904. DOI:10.1152/physrev.00041.2006.

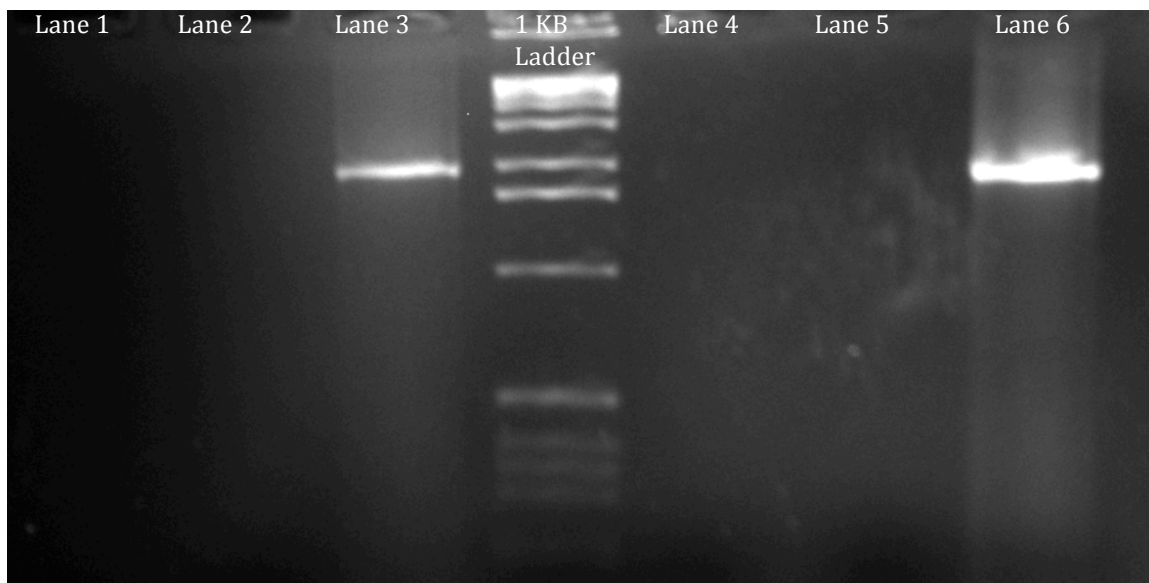
11. Pabbaraju K, Miller WL, and Sanderson KE. 2000. Distribution of intervening sequences in the genes for 23S rRNA and rRNA fragmentation among strains of *Salmonella* Reference Collection B (SARB) and SARC sets. *Journal of Bacteriology*, 182(7): 1923-1929.
12. Williams PH, Rabsch W, Methner U, Voigt W, Tschäpe H, and Reissbrodt R. 2006. Catecholate receptor proteins in *Salmonella enterica*: role in virulence and implications for vaccine development. *Vaccine*, 24(18): 3840-3844. DOI:10.1016/j.vaccine.2005.07.020.
13. Wu MT, Carlson SA, and Meyerholz DK. 2002. Cytopathic effects observed upon expression of a repressed collagenase gene present in *Salmonella* and related pathogens: mimicry of a cytotoxin from multiple antibiotic-resistant *Salmonella enterica* serotype Typhimurium phagetype DT104. *Microbial Pathogenesis*, 32(6): 279-287. DOI:10.1006/mpat.2002.0535.

**Table 1.** Summary of results for calves infected with *Salmonella* and given norepinephrine.

<i>Salmonella</i> strain	Additional treatment(s)	<i>Salmonella</i> blood culture	<i>Salmonella</i> brain culture	Neurologic signs	<i>clg</i> expression
<i>Sstp</i> NPG	None	Positive	Positive	Yes	Yes
<i>Sstp</i> NPG	Cilostazol	Positive	Negative	None	Yes
<i>Sstp</i> NPG/ pAs-Clg	None	Positive	Negative	None	No
<i>Sstp</i> SARB	None	Positive	Negative	None	No

*Salmonella* blood and brain cultures were evaluated qualitatively. Neurologic disease was ascribed if four different neurologic signs, *e.g.*, paddling, proprioceptive placing deficits, excessive ear fluttering, ataxia, tremors, or opisthotonus. *clg* expression was ascribed if an RT-PCR amplicon was observed following 40 cycles of PCR.

Figure 1. Expression of *clg* in *Sstp*NPG exposed to bovine serum. RT-PCR was performed using bacterial RNA and primers specific to *clg* in *Salmonella*. *Sstp*NPG was incubated in the presence of serum obtained from non-stressed bovine (Lane 2) and stressed cattle (Lane 3). Controls included the SARB strain incubated with serum obtained from stressed cattle (Lane 1), *Sstp*NPG incubated with norepinephrine (Lane 4) or dexamethasone (Lane 5), or *E. coli* HB101 that constitutively expresses *clg* (Lane 6).



## CHAPTER 4. BETA-LACTAM ANTIBIOTICS PREVENT *SALMONELLA*-MEDIATED ENCEPHALOPATHY REGARDLESS OF BETA-LACTAM RESISTANCE

Modification of a paper in submission to  
*The Veterinary Journal*

Nalee Xiong, Matt T. Brewer, Kristi L. Anderson, Gwyneth Watrous, Katharine E. Weeks, Alison E. Barnhill, Tim A. Day, Michael J. Kimber, and Steve A. Carlson

### ABSTRACT

Nontyphoidal *Salmonella* encephalopathies (NTSE) are a rare but serious extraintestinal manifestation of salmonellosis. Recently, we identified three unique strains of *Salmonella* that elicited neurologic disease in a bovine stress model, and this neurologic disease appears to be due to a neuroexcitation in the absence of overt brain histopathology. Recent studies suggest that beta-lactam antibiotics can exert neuroprotective effects, independent of bacteriocidal activities, by increasing glutamate export from the brain. Since glutamate is a neuroexcitant, we evaluated two bovine-approved beta-lactam antibiotics as interventions for bovine NTSE. Using a previously described norepinephrine-based stress model, calves were infected with a clinical strain of beta-lactam-resistant *Salmonella enterica* serotype Saintpaul (*Sstp*NPG) and divided into seven groups. Two groups received a beta-lactam (either ampicillin or ceftiofur) upon overt onset of salmonellosis (*e.g.* diarrhea). A third group received a beta-lactam antibiotic plus a glutamate export inhibitor. The fourth group received MK-801, a glutamate receptor antagonist, instead of an antibiotic. The fifth and sixth groups received non-beta-lactam antibiotics with either putative neuroprotective effects

(minocycline) or known bacteriocidal effects (enrofloxacin). The final group received only norepinephrine. Neurologic disease was observed in three groups - calves injected with just norepinephrine, calves co-treated with a beta-lactam and a glutamate export inhibitor, and calves treated with minocycline. Interestingly, *Salmonella* were recovered from the brains of all calves except those treated with enrofloxacin. Glutamate exporter expression was elevated in calves treated with beta-lactam antibiotics. These results support the use of beta-lactams as effective therapeutics for bovine NTSE, regardless of extended-spectrum beta-lactamase activity.

## INTRODUCTION

A clinical isolate of *Salmonella enterica* serovar Saintpaul (*Sstp*NPG) was recently implicated in an outbreak of atypical bovine salmonellosis. In this instance of non-typhoidal *Salmonella* encephalopathy (NTSE), *Sstp*NPG was isolated from Holstein calves exhibiting diarrhea, head tilt, ataxia, auditory hyper-responsiveness, and partial blindness. Under experimental conditions, signs of neuropathogenicity were reproduced in calves stressed with norepinephrine and orally challenged with *Sstp*NPG [6]. Clinical findings demonstrated that *Sstp*NPG causes bovine encephalopathy in the absence of overt histopathologic changes in the central nervous system. Additionally, only one colony-forming unit of *Sstp*NPG was recovered from each 10g of brain [6]. Therefore, the observed neurologic signs could be related to *Salmonella*-mediated neurochemical anomalies not detectable using standard histopathologic techniques.



One such neurochemical candidate is glutamate since neuroexcitement can be related to fluctuations in the release of this neurotransmitter. Activation of glutamate receptors, on both neuronal and glial cells, leads to an over-saturation of calcium influx and excess intracellular signaling (7, 16) that may not be easily detectable during the peracute phase of NTSE manifested in cattle.

To evaluate the involvement of glutamate in *Sstp*NPG-mediated neuropathogenicity, we experimentally infected calves with this isolate and treated these animals with a pharmacologic stress mediator and compounds that inhibit the effects of glutamate. Specifically, glutamate activity was targeted for inhibition by treating infected animals with either MK-801 or beta-lactam antibiotics. MK-801 is a selective and non-competitive antagonist for N-methyl-D-aspartate (NMDA) receptors, an ionotropic glutamate receptor [2, 15], while beta-lactam antibiotics were recently demonstrated to be neuroprotective by facilitating the export of glutamate from the brain via glial cells. Beta-lactam antibiotics upregulate glutamate transporter-1 (GLT-1) expression whereby GLT-1 promoter activity is enhanced in a concentration-dependent and dose-dependent manner by ceftriaxone (a third-generation cephalosporin), amoxicillin (an aminopenicillin), and a number of other beta-lactams [10].

Considering these findings, we hypothesized that either MK-801 or beta-lactam antibiotics will attenuate signs of neurologic disease in pharmacologically-stressed calves infected with *Sstp*NPG. Calves were experimentally challenged with *Sstp*NPG, administered daily doses of norepinephrine, and then treated with either a beta-lactam antibiotic, MK-801, or the combination of a beta-lactam and a glutamate export

inhibitor. Additional groups of calves received non-beta-lactam antibiotics, minocycline or enrofloxacin, capable of penetrating the blood-brain-barrier. The former was chosen because of putative neuroprotective effects on neuronal apoptosis [5, 9, 13, 14] while the latter antibiotic was selected as a control since it is not part of the antibiogram of *Sstp*NPG. These studies will determine the therapeutic value of beta-lactams versus NTSE.

## MATERIALS AND METHODS

### *Bacterial strains used and preparation*

*Sstp*NPG was obtained from calves that displayed the aforementioned clinical signs of neuropathogenicity [6]. The isolate exhibits resistance to ampicillin, florfenicol/chloramphenicol, ceftriaxone/ceftiofur, and kanamycin. This strain is sensitive to tetracyclines and fluroquinolones. Prior to challenge, *Sstp*NPG was grown statically overnight for 16 hours in LB broth containing 8 µg/mL of ceftiofur at 37°C.

Minocycline is a broad-spectrum tetracycline antibiotic that functions as a bacteriostatic antibiotic and a neuroprotective agent that inhibits apoptosis via caspase-1 and caspase-3 inhibition and MMP9 repression [5, 9, 13, 14]. More importantly, minocycline attenuates the activation of microglia, which is often associated with glutamate excitotoxicity [5, 13, 14]. As a result, we were interested in its ability to abrogate the neurologic effects of *Sstp*NPG. In order to make the *Sstp*NPG isolate minocycline-resistant, bacterial conjugation was conducted whereby minocycline-resistance conjugative plasmids were transferred from a pool of

minocycline-resistant *Salmonella* SARB strains [8] to *Sstp*NPG. Transconjugation occurred aerobically overnight at 37°C in a 1ml co-inoculum containing  $5 \times 10^8$  CFUs of *Sstp*NPG along with  $5 \times 10^7$  CFUs of each nine different minocycline-resistant SARB strains. To confirm conjugation, the liquid culture was inoculated into 50 mL of LB broth containing minocycline (16 µg/mL) and ceftiofur (8 µg/mL). Since the ceftiofur-resistance plasmid was deemed to be non-conjugative (data not shown) and thus not transferred from *Sstp*NPG to the SARB strains, the resulting transconjugates were used to infect one group of calves.

#### *In vivo experiments*

*In vivo* experiments were conducted in Holstein or Jersey calves ranging from 1 to 5 weeks of age and weighing on average 30-60 kg. Each calf received a 1 mL oral inoculum containing  $10^9$  CFUs of *Sstp*NPG, or the minocycline-resistant *Sstp*NPG isostrain, which were grown aerobically for 16 hours at 37°C. All subjects were also given daily injections of norepinephrine (20 µg/kg, IM; Sigma) to imitate stress as per McCuddin *et al.* [2008]. Three control subjects received no additional treatments while two groups of four subjects were each given a beta-lactam [either ampicillin (5 mg/kg, IM, s.i.d.; Polyflex®, Fort Dodge Laboratories) or ceftiofur (6.6 mg/kg, subcutaneous; Excede®, Pfizer)] at the onset of diarrhea. Another group of four subjects were given either ampicillin (n = 2) or ceftiofur (n = 2) along with threo-beta-hydroxyaspartate (THA; 0.1 µg/kg, IM; Sigma), a glutamate exporter inhibitor [3, 11, 12]. The effects of a glutamate receptor antagonist (MK-801; 0.4 mg/kg, IM, s.i.d.; Sigma) was also evaluated in a separate group of three calves. The final two groups of calves (three calves per

group) were given daily doses of either minocycline (10 mg/kg, IM, s.i.d.; Sigma) or enrofloxacin (10mg/kg, subcutaneous; Baytril®, Bayer). Summarization of the above can be found on Table 1.

Once subjects displayed neurological signs (3 to 6 days post infection), calves were euthanized immediately after photo-documentation of neurologic signs. Calves that did not show signs of neurologic disease were euthanized 8 to 10 days post-infection. Euthanasia was performed using xylazine (0.45 mg/kg, IM; Phoenix Laboratories) and pentobarbital (1.2 mg/kg, IV; Fort Dodge Laboratories). Animal experiments were approved by the Institutional Animal Care and Use Committee at Iowa State University (protocol 2-08-6508B).

#### *Qualitative isolation of Salmonella*

Blood (10 mL) and brain (10 g) samples were collected for the isolation of *Salmonella*. Samples were enriched in a 50 mL falcon tube and grown statically overnight at 37°C in 10 mL of LB broth with no antibiotics. *Salmonella* were selectively cultured by transferring 20 and 200 µL of LB broth culture to brilliant green agar plates containing 8 µg/mL of ceftiofur in order to isolate colonies. Following an overnight incubation at 37°C, individual colonies were sampled from each plate and identified using *Salmonella*-specific polymerase chain reaction (PCR) targeting the *sipB-sipC* junction [1].

#### *Semi-quantification of GLT-1*

RNA was isolated from brain cultures using the RNeasy Lipid Tissue Mini Kit (Qiagen). In order to quantify glutamate transporter-1 (GLT-1) expression, semi-

quantitative RT-PCR was conducted on RNA samples from calves using the following oligonucleotide primers: Glut1F 5' – CATGCACAGAGAAGGCAAAA – 3' and Glut1R 5' – AGAGTCTCCATGGCCTCTGA – 3'. Reactions were performed using Superscript III One-Step RT-PCR System with Platinum *Taq* (Invitrogen). The thermocycling program used was as follows: 55°C for 30 minutes, 94°C for 2 minutes, then either ran for 10 or 40 cycles of 94°C for 15 seconds, 45°C for 30 seconds, and 68°C for 30 seconds. Amplicons were observed at various cycles using agarose gel electrophoresis.

## RESULTS

### *Assessment of neurologic disease in SstpNPG-infected calves treated with beta-lactam antibiotics*

In order to determine if beta-lactam antibiotics could prevent *Sstp*NPG-mediated neurologic disease, calves were inoculated with *Sstp*NPG and treated with daily doses of norepinephrine. At the onset of diarrhea (one to two days post-inoculation), calves were given standard FDA-approved doses of either ceftiofur or ampicillin. Calves were then monitored for signs of neurologic disease such as seizures, opisthotonus, muscle fasciculations, ataxia, paddling, tonic-clonic movements, and excessive ear fluttering as previously described [6].

Calves that received a treatment of beta-lactam antibiotics (either ceftiofur or ampicillin) showed no signs of neurologic disease (see Figure 1). Additionally, one group of calves were administered a beta-lactam antibiotic plus THA, a glutamate

export inhibitor. This group of subjects did show signs of neurologic deficiencies in calves (see Figure 1).

*Assessment of GLT-1 expression in SstpNPG-infected calves treated with beta-lactam antibiotics*

Since beta-lactams provide neuroprotection by increasing the expression of glutamate transporters [4, 10], we semi-quantitatively assessed the expression of GLT-1 in *SstpNPG*-infected calves that were treated with either ceftiofur or ampicillin. Brains were collected following euthanasia and semi-quantitative RT-PCR was performed using mRNA and oligonucleotide primers specific to GLT-1 transcripts. When compared to expression in calves that did not receive beta-lactams, GLT-1 expression was elevated in calves treated with either ceftiofur or ampicillin (see Figure 1).

*Assessment of neurologic disease in SstpNPG-infected calves treated with a glutamate receptor antagonist*

In order to determine if the *SstpNPG*-mediated neurologic effects are associated with elevations in brain glutamate activity, *SstpNPG*-infected calves were treated with the ionotropic glutamate receptor antagonist MK-801 [2, 15]. As shown in Figure 1, MK-801-treated calves did not display neurologic signs even though *SstpNPG* was present in the brains of these calves.

*Assessment of neurologic disease in SstpNPG-infected calves treated with non-beta-lactam antibiotics*

The proposed neuroprotective effects of beta-lactams are independent of their antibacterial activities. Therefore, a control group of *SstpNPG*-infected calves were

treated with either minocycline or enrofloxacin at the onset of diarrhea. Minocycline is a neuroprotective tetracycline antibiotic that inhibits neuronal apoptosis [5, 13, 14]. Enrofloxacin was chosen since it is a bovine-approved fluoroquinolone with no known neuroprotective activities and it is not part of the *Sstp*NPG antibiogram. Neurologic disease and brain infection were noted in minocycline-treated calves whereas enrofloxacin-treated calves did not display neuropathy or brain infection (see Figure 1).

## DISCUSSION

In this study, we assessed the neuroprotective ability of beta-lactam antibiotics in a model of calves infected with *Sstp*NPG. We demonstrated that subjects given beta-lactam antibiotics did not show signs of neurologic disease despite the isolate being resistant to the antibiotics. In beta-lactam-treated calves, *Sstp*NPG could still traverse the blood-brain barrier without causing neuropathogenicity unless a glutamate export inhibitor was co-administered. Calves treated with a glutamate receptor antagonist also did not display neurologic signs. Beta-lactam-treated calves had increased expression of GLT-1, a protein that facilitates glutamate export from the brain. Therefore, it appears that *Sstp*NPG causes bovine neurologic disease through a glutamate-dependent mechanism and that beta-lactam antibiotics ameliorate these effects.

Enrofloxacin was able to prevent neuropathogenicity through conventional bactericidal activities since *Sstp*NPG was not found in the brains of calves treated with this drug. The neuroprotective effects of minocycline, however, did not alter the course

of *Sstp*NPG-mediated disease indicating that signs of neuropathogenicity may not result from NMDA excitotoxicity triggered by the activation of microglia through p38 [5, 13, 14].

In summary, our study demonstrates that the neurologic effects of *Sstp*NPG can be abolished by beta-lactam antibiotics regardless of resistance. The mechanism by which neuroprotection occurs is through the increase of glutamate transporter expression. Beta-lactams are therefore at least an adjunct therapy to be used versus bovine salmonellosis in order to prevent NTSE.

## REFERENCES

1. Carlson SA, Bolton LF, Briggs CE, Hurd HS, Sharma VK, Fedorka-Cray PJ, and Jones BD. 1999. Detection of multiresistant *Salmonella typhimurium* DT104 using multiplex and fluorogenic PCR. *Molecular and Cellular Probes*, 13(3): 213-222. DOI:10.1006/mcpr.1999.0240.
2. Halliwell RF, Peters JA, and Lambert JJ. 1989. The mechanism of action and pharmacological specificity of the anticonvulsant NMDA antagonist MK-801: a voltage clamp study on neuronal cells in culture. *British Journal of Pharmacology*, 96(2): 480-494.
3. Kojima S, Nakamura T, Nidaira T, Nakamura K, Ooashi N, Ito E, Watase K, Tanaka K, Wada K, Kudo Y, and Miyakawa H. 1999. Optical detection of synaptically induced glutamate transport in hippocampal slices. *Journal of Neuroscience*, 19(7): 2580-2588.
4. Lee SG, Su ZZ, Emdad L, Gupta P, Sarkar D, Borjabad A, Volsky DJ, and Fisher PB. 2008. Mechanism of ceftriaxone induction of excitatory amino acid transporter-2 expression and glutamate uptake in primary human astrocytes. *Journal of Biological Chemistry*, 283(19): 13116-13123. DOI: 10.1074/jbc.M707697200.
5. Matsukawa N, Yasuhara T, Hara K, Xu L, Maki M, Yu G, Kaneko Y, Ojika K, Hess DC, and Boriongan CV. 2009. Therapeutic targets and limits of minocycline neuroprotection in experimental ischemic stroke. *BioMed Central Neuroscience*, 10(126). DOI:10.1186/1471-2202-10-126.



6. McCuddin ZP, Carlson SA, and Sharma VK. 2008. Experimental reproduction of bovine *Salmonella* encephalopathy using a norepinephrine-based stress model. *The Veterinary Journal*, 175: 82-88. DOI:10.1016/j.tvjl.2006.12.014.
7. Melzer N, Meuth SG, Torres-Salazar D, Bittner S, Zozulya AL, Weidenfeller C, Kotsiari A, Stangel M, Fahlke C, and Wiendl H. 2008. A beta-lactam antibiotic dampens excitotoxic inflammatory CNS damage in a mouse model of multiple sclerosis. *Public Library of Science*, 3(9): e3149. DOI:10.1371/annotation/b898d6ee-801a-474e-9776-72e4da664b89.
8. Pabbaraju K, Miller W, and Sanderson K. 2000. Distribution of intervening sequences in the genes for 23S rRNA and rRNA fragmentation among strains of *Salmonella* Reference Collection B (SARB) and SARC sets. *Journal of Bacteriology*, 182: 1923-1929.
9. Plane JM, Shen Y, Pleasure DE, and Deng W. 2010. Prospects for minocycline neuroprotection. *Archives of Neurology*, 67(12): 1442-1448. DOI:10.1001/archneurol.2010.191
10. Rothstein JD, Patel S, Regan MR, Haenggeli C, Huang YH, Bergles DE, Jin L, Dykes-Hoberg M, Vidensky S, Chung DS, Toan SV, Bruijn LI, Su ZZ, Gupta P, Fisher PB. 2005. Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. *Nature*, 433(7021): 73-77. DOI:10.1038/nature03180.
11. Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang Y, Schielke JP, and Welty DF. 1996. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron*, 16(3): 675-686. DOI:10.1016/S0896-6273(00)80086-0.
12. Shigeri Y, Shimamoto K, Yasuda-Kamatani Y, Seal RP, Yumoto N, Nakajima T, and Amara SG. 2001. Effects of threo-beta-hydroxyaspartate derivatives on excitatory amino acid transporters (EAAT4 and EAAT5). *Journal of Neurochemistry*, 79(2): 297-302. DOI: 10.1046/j.1471-4159.2001.00588.x.
13. Tikka TM and Koistinaho JE. 2001. Minocycline provides neuroprotection against N-methyl-D-aspartate neurotoxicity by inhibiting microglia. *Journal of Immunology*, 166(12): 7527-7533.
14. Wells JE, Hurlbert RJ, Fehlings MG, and Yong VW. 2003. Neuroprotection by minocycline facilitates significant recovery from spinal cord injury in mice. *Brain*, 126(Pt7): 1628-1637. DOI: 10.1093/brain/awg178.

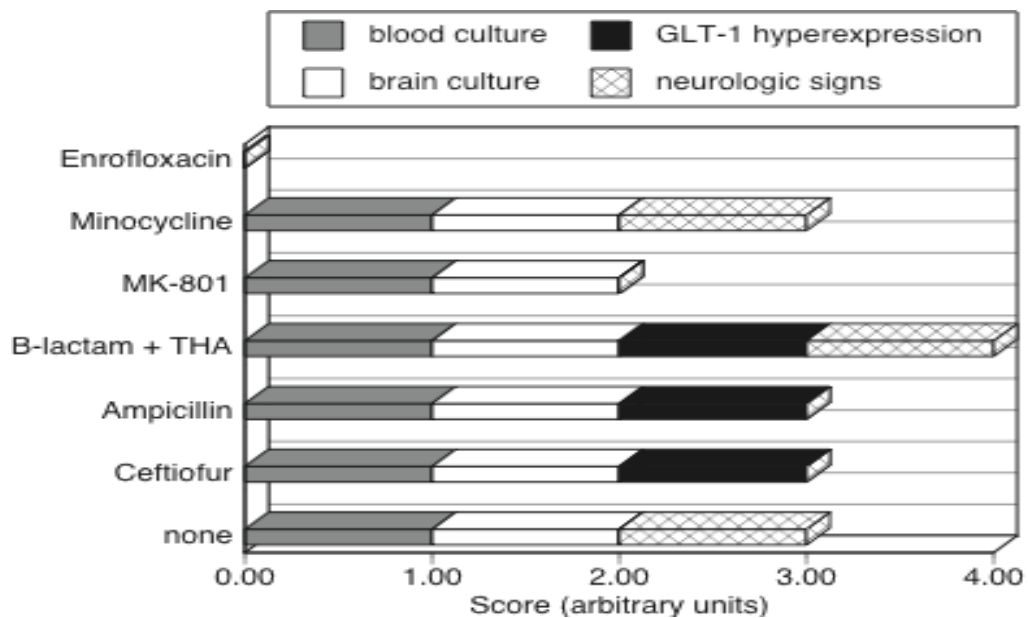
15. Wong EH, Kemp JA, Priestley T, Knight AR, Woodruff GN, and Iversen LL. 1986. The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist. *Proceedings of the National Academy of Sciences*, 83(18): 7104-7108.
16. Xin WJ, Weng HR, and Dougherty PM. 2009. Plasticity in expressin of the glutamate transporters GLT-1 and GLAST in spinal dorsal horn glial cells following partial sciatic nerve ligation. *Molecular Pain*, 5(15). DOI:10.1186/1744-8069-5-15.

Table 1. Summary of *in vivo* experimental group treatment set up.

Grps	Strain	NE	Antibiotics	GEI	n
1	<i>Sstp</i> NPG	--	--	--	3
2	<i>Sstp</i> NPG	x	--	--	4
3	<i>Sstp</i> NPG	x	Amp	--	4
4	<i>Sstp</i> NPG	x	Cf	x	4
5	<i>Sstp</i> NPG	x	Amp/Cf	x	4
6	<i>Sstp</i> NPG	x	Enro	x	3
7	<i>Sstp</i> SARB	x	--	x	4
8	<i>Sstp</i> NPG(MR)	x	Mino	--	3

NE = norepinephrine, GEI = glutamate exporter inhibitor, threo-beta-hydroxyaspartate, N = number of subjects per group, *Sstp*NTX = *Salmonella enterica* serovar Saint Paul, SARB = *Salmonella* Reference Collection B, MR = minocycline-resistant, Amp = ampicillin, Cf = ceftiofur, Enro = enrofloxacin

**Fig. 1.** Assessment of *Salmonella* blood and brain culture, GLT-1 hyperexpression, and neurologic signs in calves inoculated with *Sstp*NTX and injected with norepinephrine. Additional treatments are indicated on the y-axis: MK-801, glutamate receptor antagonist;THA, threo-beta-hydroxyaspartate, a glutamate export inhibitor. The Score is the sum of 1 arbitrary unit for positive *Salmonella* blood culture,1 arbitrary unit for positive *Salmonella* brain culture, 1 arbitrary unit for semi-quantitative detection of GLT-1 hyperexpression in brain tissues, and 1 arbitrary unit for exhibiting at least four of the following six neurologic signs: paddling, proprioceptive placing deficits, excessive ear fluttering, ataxia, tremors, or opisthotonus as per McCuddin *et al.* (2008). Each treatment group represents three or four animals.



## **CHAPTER 5. THE LIPOPOLYSACCHARIDE OF *SALMONELLA ENTERICA* SEROVAR SAINTPAUL PLAYS A ROLE IN THE INDUCTION OF NONTYPHOIDAL *SALMONELLA* ENCEPHALOPATHY IN THE BOVINE**

### **ABSTRACT**

Calves infected with *Salmonella enterica* serovar Saintpaul (*Sstp*NPG) can result in nontyphoidal *Salmonella* encephalopathy (NTSE), which produces such signs as head tilt, ataxia, seizures, and ear fluttering. Glutamate was recently established to be the element provoking these signs of neurologic disease but it is unknown what is perpetuating its release. *Sstp*NPG becomes quiescent once inside the brain, suggesting the lipopolysaccharide (LPS) of the strain could be provoking a toxic release of glutamate. In order to determine if LPS plays a role in producing the signs of NTSE, *in vivo* experiments were conducted that involved infecting calves with an LPS-free *Sstp*NPG and treating *Sstp*NPG-infected calves with polymyxin, endoserum, and MgBioserum. Debilitating LPS abolished all signs of neurologic disease in calves infected with *Sstp*NPG establishing a strong case for LPS participating in the release of glutamate-invoked NTSE.

### **INTRODUCTION**

A neuropathogenic isolate of *Salmonella enterica* serovar Saintpaul (*Sstp*NPG) that was first isolated from a 4-month old calf has been studied in order to better understand the mechanism at which it can provoke nontyphoidal *Salmonella* encephalopathy (NTSE). NTSE, which manifests as head tilt, ataxia, seizures, ear

fluttering, and other neurologic signs, rarely occurs because few nontyphoidal *Salmonella* are capable of crossing into the brain. *Sstp*NPG has been recently determined to penetrate the blood-brain barrier (BBB) paracellularly through the expression of collagenase gene (*clg*). Interestingly, *clg* is typically repressed and previous work demonstrated that bovine neuroendocrine hormones were capable of activating the expression of the gene but porcine neuroendocrine hormones were incapable of exerting similar effects [28].

Previous work indicates that glutamate is at play – blocking N-methyl-D-aspartic acid receptor (NMDAR) and increasing glutamate transporter -1 (GLT-1) expression abolished signs of neurologic disease in calves. Also, a glutamate transporter inhibitor, threo-beta-hydroxyaspartate (THA), reversed the neuroprotective effects of beta-lactam antibiotics that increase GLT-1 expression. Interestingly, treating calves that received a nonpathogenic strain of *S. Saintpaul* (*Sstp*SARB) with THA did not result in signs of neurologic disease. The aforementioned strongly signifies that a toxic release of glutamate occurs with *Sstp*NPG infection.

Excess release of glutamate is associated with such neurodegenerative diseases as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease and Huntington's disease [1, 3, 4, 7, 18, 21]. Aberrations in glutamate exportation via glutamate transporters can also result in neurodegenerative diseases [4, 14]. Therefore, glutamate is likely the candidate responsible for producing neurotoxic-like effects in calves suffering from NTSE.

Once *Sstp*NPG traverses the BBB, however, little is known about the activities that activate glutamate-based saturation NTSE. It has been established that the neurological deficits seen in calves infected with *Sstp*NPG do not result from bacteremia in cerebrovasculature or brain abscessation since only one CFU per 10 grams can be isolated from the brains of subjects [16]. There were also no distinct differences observed when conducting a hematoxylin and eosin (H & E) stain of the brain [16]. Previous work also determined that once *Sstp*NPG penetrates the BBB, it becomes transcriptionally inactive inside the brain. This indicates that whatever mechanisms are in progress the bacterial strain participates passively, highlighting the possibility of lipopolysaccharide (LPS) contributing to the detrimental release of glutamate in the system.

LPS molecules are located in the outer membrane of gram-negative bacteria and are composed of three elements: lipid A, O antigen and a core oligosaccharide [15, 24, 26]. The toxic effects of this endotoxin are a result of the lipid A, which anchors the LPS to the outer membrane of the gram-negative bacteria [15, 24, 26]. Host antibodies typically recognize the O antigen of the LPS, which often elicits a strong immune response as a result [11, 12]. Neither the O antigen or core oligosaccharide is necessary for bacterial growth; though, the two aid in antibiotic resistance, the complement system, and other environmental stresses [24]. In order to determine if the LPS of *Sstp*NPG plays a role in inducing the signs of neurologic disease seen in calves, *in vivo* experiments were conducted to identify a relationship between LPS and neurologic disease.

## MATERIALS AND METHODS

### *Bacterial strains*

*Sstp*NPG was obtained from calves that displayed clinical signs of neuropathogenicity, such as head tilt, ataxia, auditory hyper-responsiveness, partial blindness and ear fluttering [16]. Bacteria were stored in cryopreservation tubes immersed in 50% glycerol and 50% Lennox L broth at -80°C and were grown in Lennox L broth or on culture agar prior to use. Prior to challenge, *Sstp*NPG was grown statically overnight for 16 hours in a nutrient broth containing 8 µg/mL of ceftiofur at 37°C.

In order to produce bacterial strains that lacked LPS, *Sstp*NPG was grown in the presence of ethylenediaminetetraacetic acid (EDTA)(1 mM Sigma) for 2.0 hours prior to inoculation. EDTA is known to sequester the divalent cations (Mg<sup>2+</sup> and Ca<sup>2+</sup>) of the LPS thereby affecting the structural integrity and relinquishing *Sstp*NPG of LPS [2].

### *In vivo experiment*

Subjects were 1- to 5-week-old calves with an average weight of 50 kg. All animals were inoculated orally with 4.5 X 10<sup>6</sup> CFUs/kg of *Sstp*NPG except for three calves that were orally challenged with 4.5 X 10<sup>6</sup> CFUs per kilogram of *Sstp*NPG that had been treated with EDTA. In order to pharmacologically produce stress, each subject received daily injections of norepinephrine (45 µg/kg, IM, Sigma) starting on the day of inoculation and continuing until the animals were euthanized. One group of three subjects received a treatment of polymyxin nonapeptide B (1 mg/kg, IM, Sigma). Polymyxin debilitates the toxic effect of LPS by binding to and deactivating the lipid A [8, 25]. Two groups containing three subjects each received either endoserum (10



mL/lb, 1:1; IM, IMMVAC, Inc) or MgBioserum (10 mL/lb, 1:128; IM; MgBiologics) as a treatment. Both endoserum and MgBioserum contain anti-LPS antibodies that recognize, bind to, and deactivate LPS [10,19, 20]. To serve as a control for calves displaying neurologic disease, three calves received no treatment, while another group of three calves served as a positive treatment control and received ceftiofur (6.6 mg/kg, subcutaneous; Excede®, Pfizer).

Subjects were observed for signs of neurological deficits such as seizures, excessive ear fluttering, ataxia, opisthotonus, and proprioceptive placing deficits. Once they showed signs of neurologic disease (typically 4 to 9 days after inoculation), animals were euthanized. An animal with neurologic disease was defined as an animal that suffered from convulsions or that had at least 3 of the aforementioned clinical signs of neurologic deficits. Animals that did not display signs of neurologic disease were euthanized 10 days after inoculation. Euthanization of animals was conducted with xylazine (0.45 mg/kg, IM, Phoenix Laboratories) and pentobarbital (1.2 mg/kg, IV, Fort Dodge Laboratories). Animal experiments were approved by the Iowa State University Institutional Animal Care and Use Committee.

#### *Bacteriologic-based detection of SsptNPG*

Samples of the blood (10 mL) and brain (10 g) were collected from each animal for qualitative assessment of *Sspt*NPG. Samples were cultured in a 50 mL falcon tube with 10 mL of LB broth with no antibiotics and grown statically overnight at 37°C. In order to qualitatively assess the samples for *Salmonella*, 10 µL of the inoculum was plated onto a brilliant green sulfur (BGS) agar containing 8 µg/mL of ceftiofur to isolate

individual colonies. A *Salmonella*-specific PCR assay targeting the *sipB-sipC* junction was conducted on individual colonies for identification [6].

## RESULTS

In order to determine the role of LPS in producing the effects of neurological deficits seen in calves infected with *Sstp*NPG, a group of calves were inoculated with LPS-free *Sstp*NPG (*Sstp*NPG exposed to EDTA). EDTA compromises the structural integrity of LPS by sequestering divalent cations [2]. Calves treated with LPS-free *Sstp*NPG did not display signs of neurologic disease indicating that LPS may play a role in perpetuating the neurological effects (see Table 1). To further test this, two groups of calves received treatments of endoserum and Mgbioserum post-infection, which are known to bind to and deactivate LPS. As depicted in Table 1, treatments of endoserum and MgBioserum were able to ameliorate the effects of neurologic disease in calves infected with *Sstp*NPG. Another group of calves received a treatment of polymyxin nonapeptide B in order to further investigate the role of LPS in potentially provoking neurologic disease. Treatment with polymyxin nonapeptide B abolished the toxic effects of LPS by binding to and deactivating the lipid A [8, 25]. Calves that received polymyxin nonapeptide B did not show signs of neurologic disease (see Table 1). *Sstp*NPG was isolated from both the blood and brain samples of all infected calves (see Table 1).

## DISCUSSION

LPS are significant pathogen-associated molecular patterns (PAMPs) readily recognized by host antigens and thereby triggering a number of host immune responses. This endotoxin is capable of interacting with such receptors as Toll-Like Receptors and CD14 (cluster of differentiation 14), which in turn produces inflammatory responses [13, 17, 22]. Interestingly, calves infected with *Sstp*NPG do not display abnormal CNS inflammation. It is possible that the LPS of *Sstp*NPG may be triggering the release of glutamate to produce the signs of neurologic disease in calves through other means.

In order to determine if LPS does play a role in the induction of neurologic disease, calves were infected with *Sstp*NPG and then treated with pharmacological agents that inhibited the potential effects of LPS. Results indicate that treatment with polymyxin nonapeptide B, endoserum, and MgBioserum were able to ameliorate the effects of neurologic signs in calves (see Table 1). Furthermore, calves treated with LPS-free *Sstp*NPG did not display signs of neurologic disease. All calves infected with *Sstp*NPG displayed signs of systemic spread as both the blood and brain samples from each subject contained *Sstp*NPG isolates but only the control group of calves that received no treatment possessed neurological deficiencies. This indicated that despite the ability to induce systemic spread and traverse the BBB, the inhibition of the LPS definitively abolishes signs of neurologic disease. LPS may indeed play a role in perpetuating the effects that lead up to neurologic disease in calves infected with *Sstp*NPG.

LPS has been shown to trigger the release of glutamate, norepinephrine and adenosine from slices of rat parietal cortex [27]. The rapid release of glutamate due to the presence of LPS was determined to be too rapid to involve gene transcription and protein synthesis and it was also shown to be a reversible effect once LPS was removed from the system [27]. It was proposed that LPS-evoked glutamate release may be associated with immune responses such as cytokine and/or prostanoid release and may be released through glial rather than neuronal cells [27]. The release of adenosine may potentially explain why little inflammation is typically seen in calves infected with *Sstp*NPG since adenosine has been shown to decrease inflammatory responses via A<sub>2A</sub> receptors on macrophages and neutrophils [9].

It is possible that *Sstp*NPG possesses a unique structure and this possibility may give insight into which receptor is potentially being activated. Determining the uniqueness of the LPS structure could be established by introducing a nonpathogenic strain into the brain *in vivo* to determine if similar effects will ensue. Exposing calf brain to purified LPS could also offer results that could lead to a better understanding of the specificity of the LPS of *Sstp*NPG in producing neurologic disease.

## REFERENCES

1. Ahmed I, Bose SK, Pavese N, Ramlackhansign A, Turkheimer F, Hotton G, Hammers A, and Brooks DJ. 2011. Glutamate NMDA receptor dysregulation in Parkinson's disease with dyskinesias. *Brain*, [Epub ahead of print]. DOI: 10.1093/brain/awr028.
2. Alakomi HL, Saarela M, and Helander IM. 2003. Effect of EDTA on *Salmonella enterica* serovar Typhimurium involves a component not assignable to lipopolysaccharide release. *Microbiology*, 149: 2015-2021. DOI: 10.1099/mic.0.26312-0.

3. Alix JJ and Domingues AM. 2011. White matter synapses: form, function, and dysfunction. *Neurology*, 76(4): 397-404.
4. André VM, Cepeda C, Fisher YE, Huynh M, Bardakjian N, Singh S, Yang XW, and Levine MS. 2011. Differential electrophysiological changes in striatal output neurons in Huntington's disease. *Journal of Neuroscience*, 31(4): 1170-1182. DOI: 10.1523/JNEUROSCI.3539-10.2011.
5. Bradford J, Shin JY, Roberts M, Wang CE, Li XJ, and Li S. 2009. Expression of mutant huntingtin in mouse brain astrocytes causes age-dependent neurological symptoms. *Proceedings of the National Academy of Sciences of the United States of America*, 106(52): 22480-22485. DOI: 10.1073/pnas.0911503106.
6. Carlson SA, Bolton LF, Briggs CE, Hurd HS, Sharma VK, Fedorka-Cray PJ, Jones BD. 1999. Detection of multiresistant *Salmonella typhimurium* DT104 using multiplex and fluorogenic PCR. *Molecular and Cellular Probes*, 13(3): 213-222. DOI:10.1006/mcpr.1999.0240.
7. Cleva RM and Olive MF. 2011. Positive allosteric modulators of type 5 metabotropic glutamate receptors (mGluR5) and their therapeutic potential for the treatment of CNS disorders. *Molecules*, 16(3): 2097-2106. DOI:10.3390/molecules16032334.
8. Cooperstock M and Riegle L. 1981. Polymyxin B inactivation of lipopolysaccharide in vaccines of gram-negative bacteria. *Infection and Immunity*, 33(1): 315-318.
9. Cronstein BN. 1995. A novel approach to a development of anti-inflammatory agents: adenosine release at inflamed sites. *Journal of Investigative Medicine*, 43(1): 50-57.
10. Durando MM, MacKay RJ, Linda S, and Skelley LA. 1994. Effects of polymyxin B and *Salmonella typhimurium* antiserum on horses given endotoxin intravenously. *American Journal of Veterinary Research*, 55(7): 921-927.
11. Endt K, Stecher B, Chaffron S, Slack E, Tchitchek N, Benecke A, Van Maele L, Sirard JC, Mueller AJ, Heikenwalder M, Macpherson AJ, Strugnell R, von Mering C, and Hardt WD. 2010. The microbiota mediates pathogen clearance from the gut lumen after non-typhoidal *Salmonella* diarrhea. *Public Library of Sciences Pathogens*, 6(9): e1001097.

12. Forbes SJ, Eschmann M, and Mantis NJ. 2008. Inhibition of *Salmonella enterica* serovar typhimurium motility and entry into epithelial cells by a protective antilipopolysaccharide monoclonal immunoglobulin A antibody. *Infection and Immunity*, 76(9): 4137-4144.
13. Hetherington CJ, Kingsley PD, Crocicchio F, Zhang P, Rabin MS, Palis J, and Zhang DE. 1999. Characterization of human endotoxin lipopolysaccharide receptor CD14 expression in transgenic mice. *Journal of Immunology*, 162(1): 503-509.
14. Kim K, Lee SG, Kegelmann TP, Su ZZ, Das SK, Dash R, Dasgupta S, Barral PM, Hedvat M, Diaz P, Reed JC, Stebbins JL, Pellecchia M, Sarker D, and Fisher PB. 2010. Role of excitatory amino acid transporter-2 (EAAT-2) and glutamate in neurodegeneration: opportunities for developmental novel therapeutics. *Journal of Cellular Physiology*, [Epub ahead of print]. DOI: 10.1002/jcp.22609.
15. Matiasovic J, Stepanova H, Volf J, Kubala L, Ovesna P, Rychlik I, and Faldyna M. 2011. Influence of the lipopolysaccharide structure of *Salmonella enterica* serovar Enteritidis on interactions with pig neutrophils. *Veterinary Microbiology*. DOI:10.1016/j.vetmic.2011.01.007.
16. McCuddin ZP, Carlson SA, and Sharma VK. 2008. Experimental reproduction of bovine *Salmonella* encephalopathy using a norepinephrine-based stress model. *Veterinary Journal*, 175(1): 82-88. DOI: 10.1016/j.tvjl.2006.12.014.
17. Matsuguchi T, Takagi K, Musikacharoen T, and Yoshikai Y. 2000. Gene expression of lipopolysaccharide receptors, toll-like receptors 2 and 4, are differently regulated in mouse T lymphocytes. *Blood*, 95(4): 1378-1385.
18. Milanese M, Zappettini S, Onofri F, Musazzi L, Tardito D, Bonifacino T, Messa M, Racagni G, Usai C, Benfenati F, Popoli M, and Bonanno G. 2011. Abnormal exocytotic release of glutamate in mouse model of amyotrophic lateral sclerosis. *Journal of Neurochemistry*, 116(6): 1028-1042. DOI: 10.1111/j.1471-4159.2010.07155.x.
19. Morris DD, Harmon BG, and Moore JN. 1989. Evaluation of the opsonic capacity of core lipopolysaccharide antiserum of equine origin against smooth *Escherichia coli* 0111:B4, using macrophage chemiluminescence. *American Journal of Veterinary Research*, 50(8): 1272-1278.
20. Morris DD and Whitlock RH. 1987. Therapy of suspected septicemia in neonatal foals using plasma-containing antibodies to core lipopolysaccharide (LPS). *Journal of Veterinary Internal Medicine*, 1(4): 175-182.

21. Nandhu MS, Paul J, Kuruvila KP, Abraham PM, Antony S, and Paulose CS. 2011. Glutamate and NMDA receptors activation leads to cerebellar dysfunction and impaired motor coordination in unilateral 6-hydroxydopamine lesioned Parkinson's rat: functional recovery with bone marrow cells, serotonin and GABA. *Molecular and Cellular Biochemistry*, [Epub ahead of print].
22. Pålsson-McDermott EM and O'Neill LAJ. 2004. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology*, 113(2): 153-162. DOI: 10.1111/j.1365-2567.2004.01976.x.
23. Rasmussen BA, Baron DA, Kim JK, Unterwald EM, and Rawls SM. 2010.  $\beta$ -lactam antibiotic produces a sustained reduction in extracellular glutamate in the nucleus accumbens of rats. *Amino Acids*, 40(2): 761-764. DOI 10.1007/s00726-010-0589-0.
24. Raetz CR and Whitfield C. 2002. Lipopolysaccharide endotoxins. *Annual Review of Biochemistry*, 71: 635-700. DOI: 10.1146/annurev.biochem.71.110601.1355414.
25. Tsuzuki H, Tani T, Ueyama H, and Kodama M. 2001. Lipopolysaccharide: neutralization by polymyxin B shuts down the signaling pathway of nuclear factor  $\kappa$ B in peripheral blood mononuclear cells, even during activation. *Journal of Surgical Research*, 100(1): 127-134. DOI: 10.1006/jsre.2001.6227.
26. Wang X and Quinn PJ. 2010. Lipopolysaccharide: biosynthetic pathway and structure modification. *Progress in Lipid Research*, 49(2): 97-107. DOI:10.1016/j.plipres.2009.06.002.
27. Wang YS and White TD. 1999. The bacterial endotoxin lipopolysaccharide causes rapid inappropriate excitation in rat cortex. *Journal of Neurochemistry*, 72(2): 652-660. DOI: 10.1046/j.1471-4159.1999.0720652.x.
28. Xiong N, Brewer MT, Day TA, Kimber MJ, Barnhill AE, and Carlson SA. 2010. Evaluation of the pathogenicity and virulence of three strains of *Salmonella* organisms in calves and pigs. *American Journal of Veterinary Research*, 71(10): 1170-1177. DOI: 10.2460/ajvr.71.10.1170.

Table 1. *Sstp*NPG was isolated from both the blood and brain of all groups. Blockage of LPS effectively ameliorated signs of neurologic disease.

TREATMENT	BLOOD	BRAIN	NEUROLOGIC DISEASE
Ceftiofur	+	+	
None	+	+	+
Polymyxin	+	+	
EDTA	+	+	
Endoserum	+	+	
MgBioserum	+	+	



## CHAPTER 6. GENERAL CONCLUSIONS

### OVERVIEW

*Sstp*NPG is a unique isolate that is capable of producing bovine encephalopathy in the presence of host neuroendocrine hormones. Though this is not unusual for typhoidal and paratyphoidal strains, it is a rare phenomenon for nontyphoidal strains. In order to better understand *Sstp*NPG, *in vivo* and *in vitro* molecular techniques were used to determine conditions underlying neuropathogenicity, to understand how the strain traverses the BBB, to find a potential pharmacological agent that can abrogate the effects of neuropathogenicity, and to assess the bacterial factors that provoke neurologic disease.

### HOST-CONDITION SPECIFIC PHENOMENON

Stressed calves affected by *Sstp*NPG showed signs of neurologic disease, such as excessive ear fluttering and seizures. It is unknown whether these clinical findings are specific to bovine and are an indication of a distinct relationship between the host and the bacterial strain that induces nontyphoidal *Salmonella* encephalopathy (NTSE), which is a rare occurrence. In order to determine if there is a relationship between bovine and *Sstp*NPG, both porcine and bovine subjects were inoculated with *Sstp*NPG in order to compare host signs of disease.

Norepinephrine-treated pigs infected with *Sstp*NPG did not show signs of neurologic disease or systemic infection whereas calves, as expected, displayed

neurologic signs. These results indicate that there is a host condition-specific relationship that perpetuates both neurologic disease and systemic infection. The relationship between *Sstp*NPG and bovine may be more complex than host neuroendocrine hormone interactions since norepinephrine and dexamethasone were unable to provoke either neurologic disease or systemic spread in porcine. It is clear that the neuroendocrine requirements for neuropathogenicity are absent in swine.

There is most likely a distinct relationship occurring between bovine immunologic response and how *Sstp*NPG interacts with host factors. Bacterial infection often yields increased expression of cytokines, such as interferon gamma (IFN- $\gamma$ ), interleukin-10 (IL-10) and tumor necrosis factor alpha (TNF- $\alpha$ ) [26]. Subjects that show higher levels of IFN- $\gamma$ /IL-10 concurrently showed higher levels of gross pathology [26]. Cytokines are often responsible for the regulation of host antibodies. One such example is that of IFN- $\gamma$  regulating IgA, an essential constituent of cattle immune defense [7]. The release of such cytokines is typically due to lipopolysaccharides and effector proteins [8, 14, 15, 19, 25, 28]. Better understanding of the relationship between host immune response and pathogen interaction may explain the signs of infection and degree of pathogen virulence.

Protein secretion systems, T3SS-1 and T3SS-2, are most likely at play as they are the primary elements for pathogenicity and systemic spread via effector proteins. Protein secretion systems are also the most prominent way bacteria interact with their environment. Further investigation is required to better understand the interaction between *Sstp*NPG and stressed cattle. It is possible that experimentation with other

host ruminants could lead to increased understanding. Therefore, other ruminants should be considered to see if similar effects could be observed.

#### COLLAGENASE ALLOWS *Sstp*NPG TO PENETRATE BLOOD-BRAIN BARRIER

NTSE is a rare occurrence due to the fact that bacterial invasion is primarily halted prior to systemic infection and that even fewer strains possess the ability to penetrate the BBB. Previous work has pinpointed a protein with cytotoxin-like effects, later coined as collagenase (*clg*) due to its homology to a known *E. coli* collagenase [27] that may be capable of breaking down the tight junctions and the basement membrane of the BBB. *clg* is found in most *Salmonella* species and is a gene that is repressed under normal conditions.

*clg* is derepressed by bovine stress factors

In order to determine if *clg* can be derepressed in the presence of stressed bovine blood, *Sstp*NPG was grown in nutrient broth containing bovine serum from either stressed or unstressed cattle. To delineate the neuroendocrine hormones potentially involved, the strain was also grown in nutrient broth treated with norepinephrine or dexamethasone. As a control, *Sstp*SARB was also grown under the same conditions. RT-PCR results indicated that *clg* was only transcriptional active when grown in stressed bovine serum and was unable to be derepressed by norepinephrine or dexamethasone alone. Later studies indicated that exposure of *Sstp*NPG to both norepinephrine and dexamethasone was incapable of derepressing *clg* (data not shown). Though *Sstp*SARB possesses *clg*, the gene was unable to be

derepressed during any of the growth conditions. This indicates a unique relationship at play between bovine neuroendocrine factors and *Sstp*NPG.

Considering the role of *clg* *in vitro*, it is very likely that this protein is regulated by SPIs. SPI-1 is found in all species of *S. enterica* and is essential for epithelial cell invasion [20]. Though the majority of *S. enterica* possess SPI-1, effector proteins released by protein secretion systems are unique and vary from strain to strain. *clg* may be regulated by SPI-1 via T3SS-1 through the interaction with host neuroendocrine factors. Carlson and colleagues (2005) determined that SlyA represses the expression of *clg* in *S. Typhimurium* DT104. Considering this along with the fact that SlyA is a protein responsible for regulating a number of functions in *Salmonella*, it is worth pursuing to determine if this protein also represses the *clg* gene of *Sstp*NPG [4]. More importantly, further studies should be considered to determine what deactivates the repressor protein responsible for regulating the expression of *clg*.

*NTSE requires production of clg to traverse BBB*

*In vivo* experiments were conducted to determine if there is a direct relationship between *Sstp*NPG accessing the brain to that of *clg* expression. Calves were inoculated with the *Sstp*NPG strain transformed with a plasmid containing an antisense *clg* that would transiently knock out *clg* expression. Another group of calves received *Sstp*NPG as a positive control, while another set of subjects received *Sstp*SARB to serve as a negative control. All three groups received norepinephrine to pharmacologically imitate stress. Calves given the strain with a deactivated *clg* did not show signs of neurological deficits because the knock down strain was unable to penetrate the BBB.

This indicated that *clg* is pivotal for the traversal of the BBB and that the strain must access the brain to produce such effects.

*clg allows for a paracellular route into the brain*

Pharmacologically tightening the BBB hindered the strain from entering the brain. Cilostazol increases the effectiveness of tight junctions by selectively inhibiting phosphodiesterase III, the enzyme responsible for breaking down cyclic adenosine monophosphate (cAMP). Increased levels of intracellular cAMP have been shown to hinder the paracellular route into the brain by increasing tight junction complexes of the BBB [1]. This indicates that the production and release of *clg* by *Sstp*NPG results in a paracellular permeability of the BBB.

#### BETA-LACTAM ANTIBIOTICS AMELIORATE NEUROLOGIC DISEASE

Calves infected with *Sstp*NPG displayed signs of neurologic disease that included diarrhea, head tilt, ataxia, auditory hyper-responsiveness, and partial blindness. Incidentally, this form of bovine encephalopathy results in no detectable histopathologic changes in the central nervous system (*i.e.*, neuroinflammation) using standard techniques such as hematoxylin and eosin (H&E) staining [16]. McCuddin and colleagues (2008) also observed that only one CFU per 10 grams of calf brain could be isolated elucidating that the neurological deficits most likely result from *Salmonella*-mediated neurochemical abnormalities that were undetectable by standard histopathological practices. A possible candidate is the primary neurochemical excitatory transmitter, glutamate. Excitotoxicity can result from an oversaturation of

calcium influx due to increased activation of glutamate receptors. Increased calcium activity can cause excitotoxicity by triggering the activation of enzymes such as phospholipases, endonucleases, and proteases [12].

*Beta-lactam antibiotics serve as a viable neuroprotective agent*

There are five glutamate transporters responsible for the uptake of glutamate from the extracellular space: GLAST (EAAT1), GLT-1 (EAAT3), EAAC1 (EAAT3), EAAT4 and EAAT5. GLT-1 is the primary transporter that protects both neurons and glial cells from excitotoxicity and is located on glial and endothelial cells [11, 17, 22, 23].

Dysfunction of GLT-1 is associated with neurodegenerative diseases like amyotrophic lateral sclerosis and strokes [5, 10, 18]. Glutamate toxicity has also been associated with Alzheimer's disease and Huntington's disease [2, 3]. In 2005, Rothstein and colleagues determined that beta-lactam antibiotics, such as ceftriaxone, can serve as effective neuroprotective agents by increasing the expression of GLT-1 in a dose- and concentration-dependent manner.

In order to determine the ability of beta-lactam antibiotics to abolish signs of neurologic disease in calves, subjects infected with *Sstp*NPG were also treated with ampicillin or ceftiofur, which are two beta-lactam antibiotics that are FDA-approved for use in cattle. To compare to non-beta-lactam antibiotics, enrofloxacin and minocycline were also given to other subjects to determine the effectiveness of the varying mechanisms of action for each pharmacological agent. Calves given beta-lactam antibiotics were able to ameliorate effects of neurologic disease despite the strain showing *in vitro* resistance to these drugs, which was confirmed with *Salmonella*

isolates from the brain. Subjects treated with enrofloxacin also did not display neurological deficits, which resulted from the bactericidal effects of the antibiotics as confirmed by the inability to isolate *Salmonella* from either blood or brain samples.

Treatment with minocycline was ineffective despite the proposed neuroprotective effects of the drug. Minocycline blocks caspase-1 and caspase-3, thereby hindering apoptosis of cells [13, 29]. It is possible that the neurological deficits seen in calves infected with *Sstp*NPG are not due to neuronal apoptosis but rather necrosis. Due to the limitations on how long the subjects can be maintained with neurologic disease, it is difficult to see the full effects of *Sstp*NPG. Further investigation needs to be conducted to determine the physiological and phenotypical effects of the strain on calf brain.

#### *Glutamate plays a role in neurologic disease*

To further investigate the role of glutamate in this model, threo-beta-hydroxyaspartate (THA) was also given to calves that were inoculated with *Sstp*NPG and either treated with ampicillin or ceftiofur. THA is a glutamate transporter inhibitor that specifically blocks EAAT1-3. As a control, another group of calves were inoculated with the non-pathogenic strain (*Sstp*SARB) and treated with THA. Both groups were also given norepinephrine to imitate stress. Results demonstrated that THA was able to reverse the neuroprotective effects of beta-lactam antibiotics, further demonstrating that glutamate removal is critical for abolishing the signs of neurologic disease. Interestingly, blocking EAAT1-3 with THA in the control group (subjects inoculated with *Sstp*SARB) was unable to reproduce the neurological deficiencies seen in stressed

calves infected with *Sstp*NPG. This demonstrates that there is an excess release of glutamate in the system. It would be beneficial to assay the release of glutamate in subjects infected with the neuropathogenic strain when stressed to compare the quantity of glutamate with that of normally functioning subjects. However, it may be challenging to monitor free glutamate versus stored glutamate *in vivo*.

*Beta-lactam antibiotics are neuroprotective through increased GLT-1 expression*

To confirm that the neuroprotective effects of beta-lactam antibiotics are a result of increased GLT-1 expression, a semi-quantitative RTPCR was conducted whereby GLT-1 transcripts were PCR-amplified for 10 cycles and 40 cycles from brain samples extracted from subjects inoculated with *Sstp*NPG that received no treatment and from subjects inoculated with *Sstp*NPG that received either ampicillin or ceftiofur. No differences were seen at 40 cycles but at 10 cycles subjects given beta-lactam antibiotics showed an increased expression of GLT-1 confirming that neuroprotection occurs through increased GLT-1 expression.

## LIPOPOLYSACCHARIDES PROVOKE NEUROLOGIC DISEASE

Once inside the brain, little is known on the mechanisms underlying the glutamate release elicited by *Sstp*NPG. McCuddin and colleagues (2008) established that the neurologic signs seen in infected calves are not a result of colonization since they were only able to isolate one CFU per 10 grams of brain. They were also unable to establish any phenotypic anomalies of the brain through standard histopathologic techniques. Theoretically, the strain could be perpetuating glutamate release either



directly or indirectly by interacting with receptors that may trigger a signal transduction effect as we have established that blocking NMDAR with MK801 abolishes neurologic disease and that increasing the expression of GLT-1 ameliorates the effects of neurologic disease. LPS is a potential candidate since it is located on the outer membrane of gram-negative bacteria like *SstpNPG* and can readily interact with host environment including certain receptors like toll-like receptors (TLRs) and CD14 [9, 13, 21].

*LPS plays a role in the induction of neurologic disease*

What led to the hypothesis that LPS may be a potential factor in provoking the release of glutamate, which in turn is producing neurologic deficits in calves, is the status of *clg* once the strain is inside the brain. An RTPCR assay conducted on *SstpNPG* isolates from calf brain indicated that *clg* was transcriptionally inactive once the bacteria passed into the brain (See Appendix 1). This work also demonstrated that *SstpNPG* becomes quiescent once inside the brain, demonstrated by the fact that brain isolates were PCR positive for *sipB-C*, a gene complex found in all pathogenic *Salmonella*, but tested RTPCR negative for *sipB-C*. This implies that the neurologic effects of *SstpNPG* on calves are a result of a passive act, making LPS a highly feasible candidate.

In order to determine if LPS plays a role in producing the effects of neurologic disease, a group of calves were inoculated with LPS-free *SstpNPG* resulting from exposure of ethylenediaminetetraacetic acid (EDTA). EDTA destabilizes LPS by sequestering divalent cations,  $Mg^{2+}$  and  $Ca^{2+}$ , thereby compromising the structural

integrity of the lipoglycan. Two groups of calves were also infected with *Sstp*NPG and then given either endoserum or MgBioserum, which contain anti-LPS antibodies that bind to and inactivate LPS. Another group infected with *Sstp*NPG were also treated with polymyxin nonapeptide B, an antibiotic that functions similarly to a detergent by disrupting the structure of the bacterial cell membrane specifically through binding the lipid A component and thereby inactivating the toxic effects of LPS [6, 24]. Results demonstrated that debilitating the LPS of *Sstp*NPG effectively abrogates neurologic disease in calves. This denotes that LPS may be pivotal to the induction of neurologic disease by directly or indirectly releasing glutamate, the element responsible for causing deficiencies seen in infected calves.

*Potential role of LPS in producing neurologic signs*

LPS is one of the most important pathogen-associated molecular patterns (PAMPs). This lipoglycan endotoxin is often associated with triggering a majority of host immune responses particularly through the interaction with a number of receptors such as Toll-Like Receptors and CD14 (cluster of differentiation 14)[9, 13, 21]. Interestingly, these two receptors are typically associated with inflammatory responses, which were unable to be seen in calves infected with *Sstp*NPG. This indicates that LPS could be potentially interacting with another type of receptor capable of being activated by lipid ligands. *Sstp*NPG may also possess a unique LPS that confers neuropathogenicity.

## FUTURE DIRECTIONS

So far, it has been established that *Sstp*NPG traverses the BBB by releasing collagenase-like protein expressed by *clg* that is derepressed by host neuroendocrine hormones. Once inside, the strain – though transcriptionally inactive – is potentially recognized by host factors (perhaps by a lipid activated receptor) via LPS. This potentially leads to an increase of glutamate release, which then triggers the effects of neurologic deficiencies seen in calves. Beta-lactam antibiotics, though ineffective against the strain due to antibiotic resistance, are able to ameliorate such effects as ataxia, head tilt, and seizures by increasing the expression of GLT-1.

### *Investigating the effects of traversing the BBB*

Since it is not entirely known how the strain is producing the effects seen in stressed calves, it would be of interest to determine if the observed effects are unique to the strain, indicative of the host response, or possibly a result of both elements. A possible approach is to make the BBB more permeable and inoculate the calf brain with a nonpathogenic strain of *Sstp* to observe the effects of the invasion. Targeting PDE3 or adenylate cyclase in order to decrease cAMP concentrations could pharmacologically alter the BBB. Once stronger understanding of the regulatory proteins controlling *clg* is achieved, perhaps activating this silent gene can perpetuate a nonpathogenic strain to cross into the brain to compare effects to that of *Sstp*NPG.

### *Determining the role of LPS*

The results found depict that LPS plays a role in inducing the effects of neurologic disease. However, it is unknown if these effects are a result of a potentially

unique LPS composition or simply due to the presence of LPS. To better understand this, an LPS extraction from *Sstp*NPG, *Sstp*SARB, and purified LPS, which would then be put into calves, could give better insight. This would allow an improved understanding of the LPS specific to *Sstp*NPG. It would also be beneficial to determine if the biosynthesis of the LPS varies from that of the nonpathogenic *S. Saintpaul* strain. A number of enzymes responsible for the biosynthesis of LPS were cloned and sequenced to determine genetic variance and resulted in little conclusion as no difference could be found in the following genes: *galE*, *murE*, *waaL* (*rfaL*), *waaJ* (*rfaJ*), *gne*, and *MsbB*. Though there are more genes involved in the biosynthesis of LPS, it is possible that variation between the pathogenic and nonpathogenic strain can be found. Determining the structure of LPS will offer a better understanding of its role in causing neurologic disease in calves.

## REFERENCES

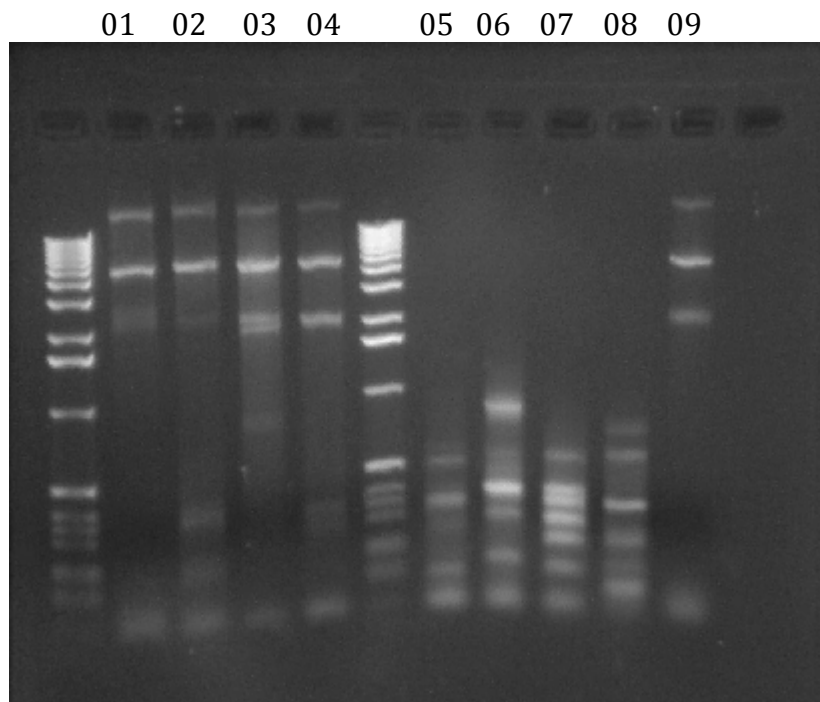
1. Adamson RH, Liu B, Nilson-Fry G, Rubin LL, and Curry FE. 1998. Microvascular permeability and number of tight junctions are modulated by cAMP. *American Journal of Physiology*, 274(6 Pt 2): H1885-1894.
2. Alix JJ and Domingues AM. 2011. White matter synapses: form, function, and dysfunction. *Neurology*, 76(4): 397-404.
3. André VM, Cepeda C, Fisher YE, Huynh M, Bardakjian N, Singh S, Yang XW, and Levine MS. 2011. Differential electrophysiological changes in striatal output neurons in Huntington's disease. *Journal of Neuroscience*, 31(4): 1170-1182. DOI: 10.1523/JNEUROSCI.3539-10.2011.
4. Carlson SA, McCuddin ZP, and Wu MT. 2005. SlyA regulates the collagenase-mediated cytopathic phenotype in multiresistant *Salmonella*. *Microbial Pathogenesis*, 38: 181-187.

5. Colton CK, Kong Q, Lai L, Zhu MX, Seyb KI, Cuny GD, Xian J, Glicksman MA, and Lin CL. 2010. Identification of translational activators of glial glutamate transporter EAAT2 through cell-based high-throughput screening: an approach to prevent excitotoxicity. *Journal of Biomolecular Screening*, 15(6): 653-662. DOI: 10.1177/1087057110370998.
6. Cooperstock M and Riegle L. 1981. Polymyxin B inactivation of lipopolysaccharide in vaccines of gram-negative bacteria. *Infection and Immunity*, 33(1): 315-318.
7. Estes DM. 2010. Regulation of IgA responses in cattle, humans, and mice. *Veterinary Immunology and Immunopathology*, 138(4): 312-317. DOI:10.1016/j.vetimm.2010.10.009.
8. Harada K and Nakanuma Y. 2010. Biliary innate immunity: function and modulation. *Mediators of Inflammation*, pii: 373878. DOI: 10.1155/2010/373878.
9. Hetherington CJ, Kingsley PD, Crocicchio F, Zhang P, Rabin MS, Palis J, and Zhang DE. 1999. Characterization of human endotoxin lipopolysaccharide receptor CD14 expression in transgenic mice. *Journal of Immunology*, 162(1): 503-509.
10. Kim K, Lee SG, Kegelmann TP, Su ZZ, Das SK, Dash R, Dasgupta S, Barral PM, Hedvat M, Diaz P, Reed JC, Stebbins JL, Pellecchia M, Sarker D, and Fisher PB. 2010. Role of excitatory amino acid transporter-2 (EAAT2) and glutamate in neurodegeneration: opportunities for developing novel therapeutics. *Journal of Cellular Physiology*, [Epub ahead of print]. DOI: 10.1002/jcp.22609.
11. Lee SG, Su Z-Z, Emdad L, Gupta P, Sarkar D, Borjabad A, Volsky DJ, and Fisher PB. 2008. Mechanism of ceftriaxone induction of excitatory amino acid transporter-2 expression and glutamate uptake in primary human astrocytes. *Journal of Biological Chemistry*, 283(19): 13116-13123. DOI: 10.1074/jbc.M707697200.
12. Manev H, Favaron M, Guidotti A, and Costa E. 1989. Delayed increase in Ca<sup>2+</sup> influx elicited by glutamate: role in neuronal death. *Molecular Pharmacology*, 36(1): 106-112.
13. Matsuguchi T, Takagi K, Musikacharoen T, and Yoshikai Y. 2000. Gene expression of lipopolysaccharide receptors, toll-like receptors 2 and 4, are differently regulated in mouse T lymphocytes. *Blood*, 95(4): 1378-1385.
14. McAleer JP and Vella AT. 2010. Educating CD4 T cells with vaccine adjuvants: lessons from lipopolysaccharide. *Trends in Immunology*, 31(11): 429-435. DOI:10.1016/j.it.2010.08.005.

15. McCoy-Simandle K, Stewart CR, Dao J, Debroy S, Rossier O, Bryce PJ, and Cianciotto NP. 2011. *Legionella pneumophila* type II secretion dampens the cytokine response of infected macrophages and epithelia. *Infection and Immunity*, [Epub ahead of print].
16. McCuddin ZP, Carlson SA, and Sharma VK. 2008. Experimental reproduction of bovine *Salmonella* encephalopathy using a norepinephrine-based stress model. *The Veterinary Journal*, 175: 82-88. DOI:10.1016/j.tvjl.2006.12.014.
17. Melzer N, Meuth SG, Torres-Salazar D, Bittner S, Zozulya AL, Weidenfeller C, Kotsiari A, Stangel M, Fahlke C, Wiedl H. 2008. A  $\beta$ -lactam antibiotic dampens excitotoxic inflammatory CNS Damage in a mouse model of Multiple Sclerosis. *Public Library of Science One*, 3(9): e3149. DOI: 10.1371/journal.pone.0003149.
18. Milanese M, Zappettini S, Onofri F, Musazzi L, Tardito D, Bonifacino T, Messa M, Racagni G, Usai C, Benfenati F, Popoli M, and Bonanno G. 2011. Abnormal exocytotic release of glutamate in mouse model of amyotrophic lateral sclerosis. *Journal of Neurochemistry*, 116(6): 1028-1042. DOI: 10.1111/j.1471-4159.2010.07155.x.
19. Monroe KM, McWhirter SM, and Vance RE. 2010. Induction of type I interferons by bacteria. *Cellular Microbiology*, 12(7): 881-890. DOI: 10.1111/j.1462-5822.2010.01478.x
20. Morgan, E. 2007. Chapter 4: *Salmonella* pathogenicity islands. *Salmonella Molecular Biology and Pathogenesis*. Horizon Biosciences, 67 – 88.
21. Pålsson-McDermott EM and O'Neill LAJ. 2004. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology*, 113(2): 153-162. DOI: 10.1111/j.1365-2567.2004.01976.x.
22. Rasmussen BA, Baron DA, Kim JK, Unterwald EM, and Rawls SM. 2010.  $\beta$ -lactam antibiotic produces a sustained reduction in extracellular glutamate in the nucleus accumbens of rats. *Amino Acids*, 40(2): 761-764. DOI 10.1007/s00726-010-0589-0.
23. Rothstein JD, Patel S, Regan MR, Haenggell C, Huang YH, Bergles DE, Jin L, Dykes-Hoberg M, Vidensky S, Chung DS, Vang-Toan S, Bruijn LI, Su Z-z, Gupta P, and Fisher PB. 2005.  $\beta$ -lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. *Nature*, 433(7021): 73-77. DOI:10.1038/nature03180.

24. Tsuzuki H, Tani T, Ueyama H, and Kodama M. 2001. Lipopolysaccharide: neutralization by polymyxin B shuts down the signaling pathway of nuclear factor  $\kappa$ B in peripheral blood mononuclear cells, even during activation. *Journal of Surgical Research*, 100(1): 127-134. DOI: 10.1006/jsre.2001.6227.
25. Viboud GI and Bliska JB. 2005. *Yersinia* outer proteins: role in modulation of host cell signaling responses and pathogenesis. *Annual Review of Microbiology*, 59: 69-89. DOI: 10.1146/annurev.micro.59.030804.121320
26. Witchell J, Maddipatla SV, Wangoo A, Vordermeier M, and Goyal M. 2010. Time dependent expression of cytokines in *Mycobacterium bovis* infected cattle lymph nodes. *Veterinary Immunology and Immunopathology*, 138(1-2): 79-84. DOI:10.1016/j.vetimm.2010.07.004.
27. Wu MT, Carlson SA, and Meyerholz DK. 2002. Cytopathic effects observed upon expression of repressed collagenase gene present in *Salmonella* and related pathogens: mimicry of a cytotoxin from multiple antibiotic-resistant *Salmonella enterica* serotype Typhimurium phagetype DT104. *Microbial Pathogenesis*, 33: 279-287. DOI: 10.1006/mpat.2002.0535.
28. Yen H, Ooka T, Iguchi A, Hayashi T, Sugimoto N, and Tobe T. 2010. NleC, a type III secretion protease, compromises NF- $\kappa$ B activation by targeting p65/RelA. *Public Library of Sciences*, 16(6): e1001231. DOI: 10.1371/journal.ppat.1001231.
29. Zhu S, Stavrovskaya IG, Drozda M, Kim BYS, Ona V, Li M, Sarang S, Liu AS, Hartley DM, Wu DC, Gullans S, Ferrane RJ, Przedborski S, Kristal BS, and Friedlander RM. 2002. Minocycline inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in mice. *Nature*, 417(6884): 74-78. DOI:10.1038/417074.

## APPENDIX. TRANSCRIPTIONAL ACTIVITY OF *CLG* INSIDE THE BRAIN



The image is an RTPCR cloning for *clg* that shows collagenase is only transcriptionally active in *Salmonella* isolated from the blood (lane 01 – 04) but not from the brain (lane 05-08) as depicted when compared to the positive control (lane 09). This alludes to the strain becoming dormant after passage into the brain.